Studies on food material extraction and characteristics from sword bean

（ナタマメからの食素材の抽出と特性に関する研究）

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Contents

Abbreviations

PAGE	Polyacrylamide gel electrophoresis
RD	Drilled RSB
RM	Milled RSB
RSB	Red sword bean
SDS	Sodium dodecyl sulfate
Tris	2-Amino-2-hydroxymethyl-1,3-propanediol
WD	Drilled WSB
WM	Milled WSB
WSB	White sword bean
WU	Untreated WSB

General introduction

Protein supply source currently depends on domestic animal meat to a large extent, we thus took multiple animal-based foods. However, recent studies showed that the consumption of animal-based foods increases the risk of cancer (Kurahashi *et al*., 2008; Youngman and Campbell, 1992; Horio *et al*., 1991). In addition, it is extrapolated that social phenomena such as urbanization, population growth, and the global rising of the middle class lead to an imbalance of demand versus greater animal meat consumption supply in the world's immediate future (CB Insights Research, 2019). However, the consumption of plant-based foods leads to an increased intake of dietary fiber, leading to the prevention of lifestyle-related diseases (Lairon *et al*., 2005). Moreover, it was also reported that plant-based food intake could inhibit the development of foci (Schulsinger *et al*., 1989) and decrease the risk of cancer (Badger *et al*., 2005). These reports imply that plant-based food intake is important for a healthy lifestyle.

Currently, we depend on soybean as the most frequent source of plant-based foods. Soybean exhibits a high protein content (approximately 33.8%) (MEXT, 2015) and processing characteristics, it is thus used for multiple soy products, soymilk, tofu, and miso. Recently, soybean was used as a material for oils and fats, covering approximately 90% of the soybean consumption (MAFF, 2019). The oils and fats from soybeans are used as biodiesel fuel, an alternative to gas oil for environmental conservation. In addition, the number of health-conscious consumers increases, who prefer plant-based foods, soybeans, and soy products in Japan. Therefore, soybean is in great demand. However, its yield varies significantly due to the climate change caused by the global warming and extreme weather (MAFF, 2019). From an imbalance of demand versus supply, the

international soybean price remains high. As a possible solution to these problems, I considered the use of beans other than soybean as a supply for plant-based foods. From the nutritional and agricultural characteristics presented below, I selected sword bean (*Canavalia gladiata*) as the first candidate for the development of new plant-based foods.

Sword bean (*Canavalia gladiata*) is a leguminous plant originating in the Asian tropics and subtropics (Bressani *et al*., 1987; Siddhuraju and Becker, 2001). This plant grows even at high temperatures (15-30°C), and its average yield is comparable to that of soybean (Bressani *et al*., 1987). Moreover, it is relatively resistant to pests and diseases (Smartt, 1976). Regarding nutritional properties, sword bean contains approximately 25.5% protein, 3.3% fat, and 61.8% carbohydrate (Vadivel and Janardhanan, 2005). In addition, recent animal experiments suggested that sword bean seed consumption might prevent postmenopausal osteoporosis (Byun and Lee, 2010) and that extracts prepared with 50% ethanol inhibit the *Porphyromonas gingivalis* infection-induced alveolar bone resorption (Nakatsuka *et al*., 2014). The white sword bean has been eaten as a green vegetable in Asia and used as a medicinal plant source of traditional Chinese medicine in many Eastern Asian countries (Purseglove, 1968). In Japan, sword bean-containing pods have been used for industrial supplies, teas, or different kinds of toothpastes. However, sword bean seeds have rarely been used for processed foods.

As described above, a new source of plant-based foods would be much required. Based on the agricultural and nutritional features of sword bean, it could be potentially used for foods. Nevertheless, the reason for unutilized bean is the lack of studies about processing characteristics and nutritional components of sword bean. In this study, I attempted to extract nutritional components to use them as sword bean-derived food materials and examined their physicochemical properties. In Chapter 1, an optimal

method for processing white sword beans (*C. gladiata alba* MAKINO) was established, and sword bean proteins were extracted. Furthermore, the effect of heating or $MgCl_2$ supplementation on sword bean proteins was examined. In Chapter 2, the relationship between the concentration of divalent metal ions and canavalin solubility was investigated. In Chapter 3, the effect of pretreatments on protein extraction and protein properties of white sword bean and red sword bean (*C. gladiata var.* gladiata) were discussed. Chapter 4 presents the salt concentration-dependent structural transitions of canavalin. In Chapter 5, gelation substances were extracted from white sword bean and gelation conditions and characteristics were examined. The findings of this study could potentially contribute to the more extended use of sword bean in the food industry.

Chapter 1

Precipitation of sword bean proteins by heating and addition of magnesium chloride in a crude extract

1.1. Introduction

White sword beans (*Canavalia gladiata*) have traditionally been eaten in the Asian tropics and subtropics (Bressani *et al.*, 1987; Siddhuraju and Becker, 2001) and are used as a source of Chinese medicines in many East Asian countries. Dried sword bean seeds were used in ancient times as a food or forage crop in the Southwestern United States, Mexico, and Central America countries (Sauer and Kaplan, 1969). Recently, sword bean seeds and pods have also been used to make tea in Japan. From an agricultural perspective, the average yield of sword beans is comparable to that of soybeans under optimal agricultural management conditions (Bressani *et al.*, 1987). In addition, sword beans are relatively resistant to pests and diseases (Smartt, 1976). These applications and agricultural characteristics of sword beans suggest their potential use in the production of processed foods.

The crude protein content of sword beans is slightly lower than that of soybeans (Bressani *et al.*, 1987; Rajaram and Janardhanan, 1992; MEXT, 2015; Sasipriya and Siddhuraju, 2013). Thus, sword beans may be used as an alternative protein source after proper cooking and processing (Rajaram and Janardhanan, 1992). Interestingly, some recent animal experiments have suggested that consumption of sword bean seeds may prevent postmenopausal osteoporosis (Byun and Lee, 2010) and that extracts prepared with 50% ethanol inhibit alveolar bone resorption induced by Porphyromonas gingivalis infection (Nakatsuka *et al.*, 2014). Despite its nutritional, functional, and production

potential, the seeds of sword beans have not been used for the production of processed foods as an alternative protein source, partly because our lack of understanding of the characteristics of sword bean proteins.

Some sword bean proteins have been analyzed in various biochemical studies. For example, proteins have been extracted from ground whole mature seeds in a high-concentration (0.15 M) NaCl solution (Moreira and Cavada, 1984; Ceccatto *et al.*, 2002; Moreno *et al.*, 2004; Delaorre *et al.*, 2007; Bezerra *et al.*, 2007). However, because protein extracts containing high concentration of salts are inadequate for the production of processed foods, this method may not be applied for commercial purposes. Therefore, in order to use sword beans in the production of processed foods, novel methods are needed for the optimal extraction of sword bean proteins.

In this study, it is established a novel method for the extraction of sword bean proteins from dried seeds in distilled water. In addition, it is examined whether heating and the addition of $MgCl_2$ could be used to precipitate protein from crude extracts. This study provides important insights into the production of processed foods from sword beans, and can thus enable sword beans to be used as potential alternative protein sources.

1.2. Materials and Methods

1.2.1. Materials

Dried white sword beans and soybeans (cultivar Fukuyutaka) were purchased from Koyamaen (Hyogo, Japan) and Tomizawa Corporation (Tokyo, Japan), respectively. General chemical reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

1.2.2. Determination of soaking time

Soaking time was determined by measuring the bean size and absorbed water weight. Beans were soaked in 10 volumes (v/w) of distilled water at 20°C for various durations. Bean size was measured with micrometer calipers (VC-15, ASONE, Osaka, Japan). The ratio was calculated by dividing the size of soaked beans by that of dried beans. The weight of beans was measured using an electronic balance (HR-120, A&D Company, Tokyo, Japan). The weight of the absorbed water was estimated by subtracting the initial weight of dried beans from that of soaked beans. The ratio was calculated by dividing the weight of the absorbed water by the initial weight of the dried beans. Data are represented as the average ± standard deviation of 10 beans.

1.2.3. Preparation of sword bean extracts

Soaked sword beans were ground in eight volumes (v/w) of distilled water with a hand blender (CSB-77JBSTRW, Cuisinart, CT, USA) on ice for 5 min. In a manipulation, which is generally used for making soymilk, a suspension containing ground beans was heated at 100°C for 3 min and then separated into an extract (Extract A) and waste by squeezing with a cotton cloth. In another manipulation, the suspension was squeezed with the same cotton cloth to be separated into an extract (Extract B) and waste. Furthermore, Extract B was heated at 100°C for 3 min and then separated into an extract (Extract C) and waste by squeezing with the same cotton cloth. The experimental scheme for the manipulation is shown in Figure 1.1.

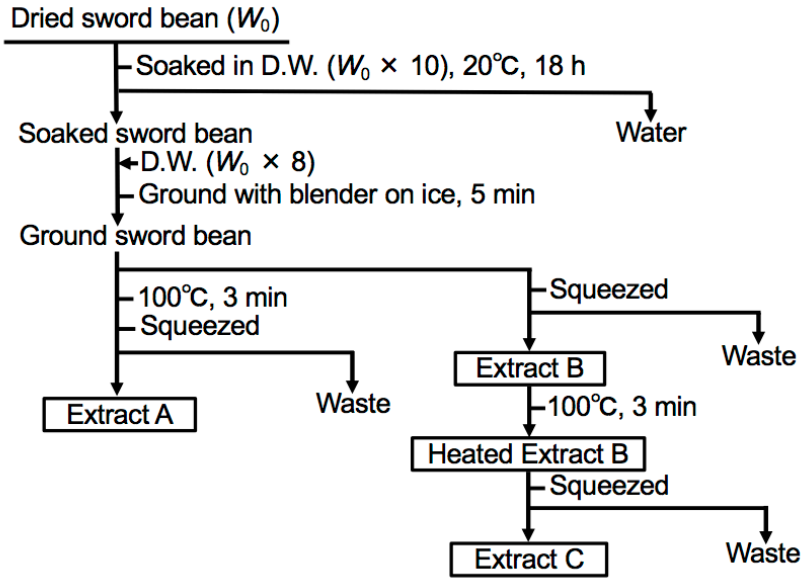

Figure 1.1 Experimental scheme for preparation of sword bean extracts.

W_0 shows the initial weight of dried sword beans. Boxes show the sword bean extracts.

1.2.4. Measurement of protein concentration

Protein concentrations were determined using the Bradford method with reagents from Bio-Rad Laboratories Inc. (CA, USA), using bovine serum albumin as a standard. Data are represented as the average ± standard deviation of three independent experiments.

1.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with 12.5% polyacrylamide gels at a constant current of 12.5 mA for 2.5 h according to standard methods described by Laemmli (1970). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250. Samples were mixed with 0.33 volumes of an SDS buffer (0.25 M Tris-HCl at pH 7.0 containing 4% SDS, 5% 2-mercaptoethanol, and 40% glycerol). Prior to SDS-PAGE, the samples were incubated in

boiling water for 5 min. The molecular weight standard was purchased from Life Technologies Japan Ltd. (Tokyo, Japan).

1.2.6. Determination of the precipitation efficiency following heating

Extract B was separated into the supernatant and precipitate by centrifugation at 9,100 × *g* at 4°C for 10 min. The supernatant was used as an initial sample for determining the precipitation efficiency. The sample was then heated at various temperatures for 20 min followed by incubation on ice for 5 min. The sample was separated into the supernatant and precipitate by centrifugation, as described for preparation of the initial sample. Supernatant proteins were quantified as described above. The ratio of protein concentration was expressed as the percentage of the supernatant protein concentration of the heated sample to that of the initial sample. Wet precipitate weight was measured with an electronic balance (HR-120, A&D Company). The precipitation efficiency was expressed as the percentage of the weight of the wet precipitate to that of the initial sample. Data represent the average of three independent experiments.

1.2.7. Determination of the precipitation efficiency following addition of magnesium chloride

The initial sample was prepared as described above. The sample was incubated at 25°C, 50°C, 75°C, or 100°C, respectively, for 5 min. Next, 0.11 volumes of 200 mM $MgCl_2$ or distilled water was added to the incubated sample while mixing, and the samples were then incubated again at the same temperature for 15 min. After heating, the mixtures were incubated on ice for 5 min and then separated into supernatants and precipitates by centrifugation at 9,100 × *g* for 20 min at 4°C. The precipitation efficiency was expressed

as the percentage of the wet weight to that of the initial sample. Supernatant protein concentrations were quantified as described above. The ratio of protein concentration was expressed as the percentage of the supernatant protein concentration of the sample with added $MgCl_2$ to that of the sample with distilled water only. Data represent the average of three independent experiments.

1.2.8. Analysis of the N-terminal amino acid sequence

After SDS-PAGE, proteins were transblotted onto polyvinylidene difluoride membranes (GE Healthcare Japan, Tokyo, Japan). The N-terminal amino acid sequence was determined with a protein sequenator (Procise 492, Life Technologies Japan Ltd).

1.2.9. Statistical analyses

A one-way analysis of variance and Bartlett test were used to compare the means among groups in the experiment for the protein concentration of the extract. A two-way analysis of variance and Bartlett test were used to compare the means among groups in the experiment for the effect of heating and adding $MgCl_2$. A *post hoc* analysis was performed with the Tukey-Kramer test if the analysis of variance had revealed significance. Differences with $p < 0.05$ are considered to be significant in these statistical analyses.

1.3. Results

1.3.1. Size and weight of white sword beans

White sword beans were much larger than soybeans in appearance (Figure 1.2A). The sizes and weights of dried beans are summarized in Table 1.1. The minor axis size and thickness of sword beans was approximately 1.7 times larger than that of soybeans, while

the long axis size was about 3.1 times larger than that of soybeans. Furthermore, sword beans weighed about 7.8 times more than soybeans. The standard deviations demonstrated that there were only minor differences between individual sword beans.

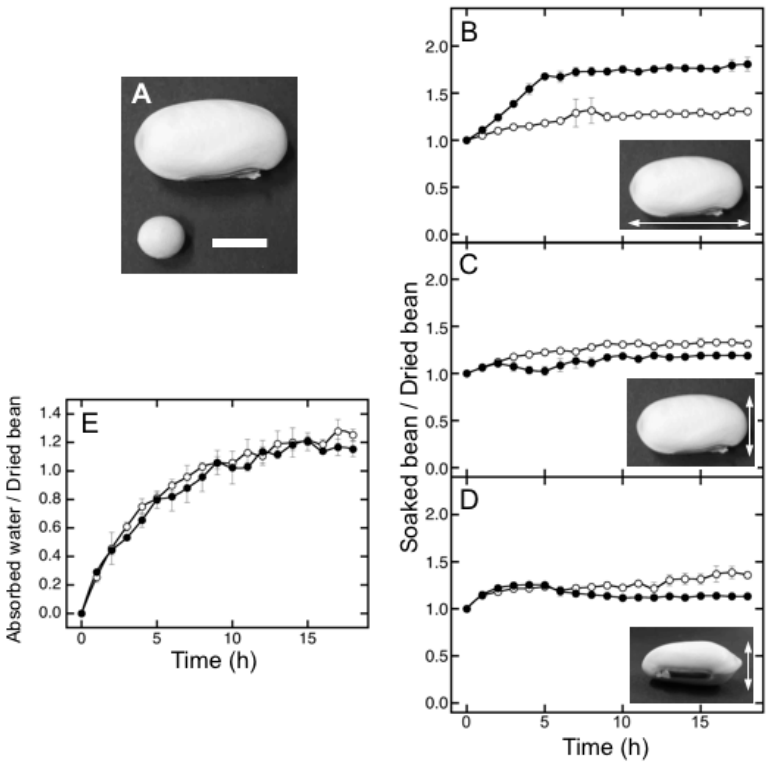

Figure 1.2 Size transition of sword beans and weight transition of absorbed water during soaking.

(A) Comparison of sword beans (upper) and soybeans (lower). The white bar is 1 cm. Sword beans (open circles) and soybeans (closed circles) were soaked in 10 volumes (v/w) of distilled water (B-E). The long axis (B), minor axis (C), and thickness (D) of beans were measured with micrometer calipers. Data represent the average ± standard deviation of 10 beans. The intersection represents the direction of the size measurement. The double-headed arrows show the long axis (B), minor axis (C), and thickness (D). The weight of the absorbed water was estimated by subtracting the initial weight of the dried beans from that of the soaked beans (E). The ratio was calculated by dividing the weight of the absorbed water by the initial weight of the dried beans. Data represent the average ± standard deviation of 10 beans.

Table 1.1 Comparison of the size and weight between dried beans.

	Long axis (cm)	Minor axis (cm)	Thickness (cm)	Weight (g)
Soybean	0.88 ± 0.03	0.83 ± 0.02	0.70 ± 0.04	0.32 ± 0.02
Sword bean	2.76 ± 0.12	1.39 ± 0.07	1.19 ± 0.04	2.48 ± 0.27

Data represent the average ± standard deviation of 10 beans selected at random.

1.3.2. Bean size and absorbed water weight

Soaking time is an important factor in the production of processed foods from dried beans. Therefore, it is determined the optimal time for soaking of beans prior to processing. When beans had absorbed a sufficient amount of water, the size and weight would plateau. To determine the soaking time required for sufficient water absorption, it is analyzed the changes in bean size and absorbed water weight for sword beans and compared these data with soaking of soybeans (Figure 1.2B-E).

Sword bean size increased slowly and nearly reached a plateau after 16 h (Figure 1.2B-D). In the long and minor axis directions, the size was approximately 1.3 times greater than that of dried beans (Figure 1.2B and C). For the thickness measurements, the size was approximately 1.4 times greater than that of dried beans (Figure 1.2D). For soybeans, the length of the long axis markedly increased at a soaking time of 5 h and nearly reached a plateau, measuring approximately 1.8 times greater than that of dried soybeans (Figure 1.2D). Additionally, the length of the minor axis measured 1.1 times greater than that of dried beans at 2 h and then decreased to the same size of dried beans at 5 h; after 5 h, the minor axis size slowly increased by approximately 1.2-fold (Figure

1.2C). The thickness of soybeans was approximately 1.2 times greater than that of dried beans by 2 h, plateaued, and then decreased (Figure 1.2D). These results demonstrated that the size of soybeans inconstantly changed, in contrast to the gradual and continual increase in size of sword beans.

The weight of absorbed water in sword beans increased and nearly reached a plateau at 13 h, similar to that in soybeans (Figure 1.2E). At 18 h, the weight of absorbed water increased by approximately 1.3-fold (Figure 1.2E, open circles). These results indicated that sword beans absorbed 1.3 volumes of water relative to the weight of dried beans. From the observed changes in sword bean size and weight, it is concluded that sword beans absorbed sufficient water after soaking for at least 16 h. Therefore, in subsequent experiments, sword beans were soaked for 18 h.

1.3.3. Extraction of sword bean proteins

For the extraction of sword bean proteins from soaked beans, sword beans were ground in distilled water with a blender. The suspension containing ground beans was heated at 100°C and then squeezed (Extract A), a method similar to the traditional preparation of soymilk. Extract A was a slightly clouded solution (Figure 1.3A, a). The protein concentration of Extract A was 1.2 ± 0.2 mg/mL (Figure 1.3B), which was much lower than expected, suggesting that heating at 100°C induced protein precipitation. The same suspension was therefore squeezed without heating (Extract B) and then heated at 100°C followed by squeezing (Extract C). Extract B had a watery milky appearance (Figure 1.3A, b) and exhibited a protein concentration of 15.9 ± 1.9 mg/mL (Figure 1.3B). During the preparation of Extract C, the protein from Extract B precipitated after heating at 100°C (Figure 1.3A, c). Extract C was also a slightly clouded solution; it looked similar to

Extract A (Figure 1.2A, d) and exhibited a protein concentration of 0.8 ± 0.1 mg/mL (Figure 1.3B). Thus, the protein concentration of Extract B was more than 13-fold higher than that of Extract A and Extract C (Figure 1.3B). Statistical analysis also indicated that the protein concentration of Extract B was significantly higher than that of Extract A and Extract C ($p < 0.001$) and that the concentration was not significantly different between Extract A and Extract C ($p > 0.5$). As described above, proteins in Extract B were precipitated by heating at 100°C (Figure 1.3A, c). The precipitated proteins were collected by squeezing with a cotton cloth (Figure 1.3C). Approximately 8.6 g of wet precipitate was obtained from 100 g of Extract B. These results suggested that most sword bean proteins were precipitated by heating at 100°C.

The sword bean proteins contained in Extract B were separated on an SDS-polyacrylamide gel (Figure 1.3D). SDS-PAGE analysis indicated that the crude extract of soybean (lane1) and sword bean (lanes 2 and 3) contained a variety of proteins (lane 2). However, the band pattern of sword bean proteins (lane 2) was completely different from that of soybean proteins (lane 1) for an equivalent amount of protein (10 μg per lane). The band pattern also showed that Extract B contained high levels of proteins having molecular weights of approximately 48 and 29 kDa (Figure 1.3D, lanes 2 and 3). From these results, I chose to use Extract B for subsequent physicochemical analyses to determine the properties of sword bean proteins.

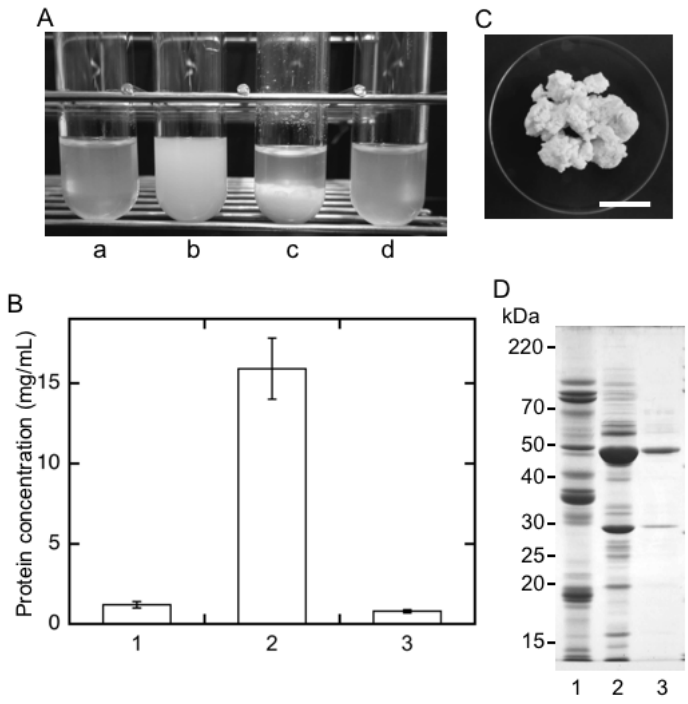

Figure 1.3 Protein extraction from sword beans.

(A) Extract A was prepared by heating followed by squeezing (a). Extract B was prepared by squeezing without heating (b). Extract B was heated (c) and then squeezed (d) for the preparation of Extract C. (B) The protein concentrations of Extract A (1), Extract B (2), and Extract C (3) were determined using the Bradford method with bovine serum albumin as a standard. Data represent the average ± standard deviation of three independent experiments. (C) The precipitate was collected by heating Extract B in the preparation of Extract C. The white bar represents 2 cm. (D) Sword bean proteins were separated on an SDS-polyacrylamide gel (12.5% polyacrylamide). Proteins corresponding to 10 μg of soybean protein (lane 1), 10 μg of sword bean protein from Extract B (lane 2), and 1 μg of sword bean protein from Extract B (lane 3) were electrophoresed on an SDS-polyacrylamide gel (12.5% polyacrylamide) and stained with Coomassie Brilliant Blue R-250.

1.3.4. Heat-dependent precipitation of sword bean proteins

Most sword bean proteins were precipitated by heating at 100°C (Figure 1.3B). I therefore investigated the thermal dependency of the precipitation of sword bean proteins. In the preparation of Extract B, I roughly leached the sword bean suspension with a cotton cloth

as described above. For further removal of residual precipitable substances from Extract B, the extract was separated into supernatants and precipitates by centrifugation. The supernatants were incubated at various temperatures and then were further separated into supernatants and precipitates by an additional centrifugation. The concentration of protein in the supernatant and the wet precipitate weight were measured for evaluation of heat-dependent precipitation (Figure 1.4A). The ratio of the protein concentration increased slightly from 25°C to 40°C (Figure 1.4A, open circles). Thus, mild heating may only slightly increase the solubility of sword bean proteins in the extract. The ratio of the protein concentration decreased slightly at temperatures ranging from 40°C to 75°C and then decreased dramatically at 90°C (Figure 1.4A, open circles). This dramatic decrease suggested that most sword bean proteins were precipitated by heating at more than 90°C. In contrast, the precipitation efficiency increased substantially at 55°C, followed by a slight increase at temperatures ranging from 60°C to 75°C and a dramatic increase again at temperatures ranging from 80°C to 90°C (Figure 1.4A, closed circles). These temperature-dependent changes in protein concentration were roughly consistent with changes in precipitate formation. However, at 55°C, there were clear differences between protein concentration and precipitate weight. This may be explained by the presence of a non-proteinaceous polymer, such a polysaccharide, following heating at 55°C.

SDS-PAGE analysis provided visualization of the heat-dependent behaviors of sword bean proteins in the supernatant (Figure 1.4B). Removal of residual precipitable substances from Extract B only slightly influenced the band pattern (Figure 1.4B, lanes 1 and 2). Minor protein bands with molecular weights of approximately 20, 27, 33, and 55 kDa were decreased by heating at less than 75°C (Figure 1.4B, lanes 3–6). Major protein bands (48 and 29 kDa) were largely decreased in supernatants by heating at 100°C (Figure

1.4B, lane 7). Some minor protein bands were not completely disrupted by heating at 100°C (Figure 1.4B, lane 7). These results were consistent with the results shown in Figures 1.3, B and 1.4, A.

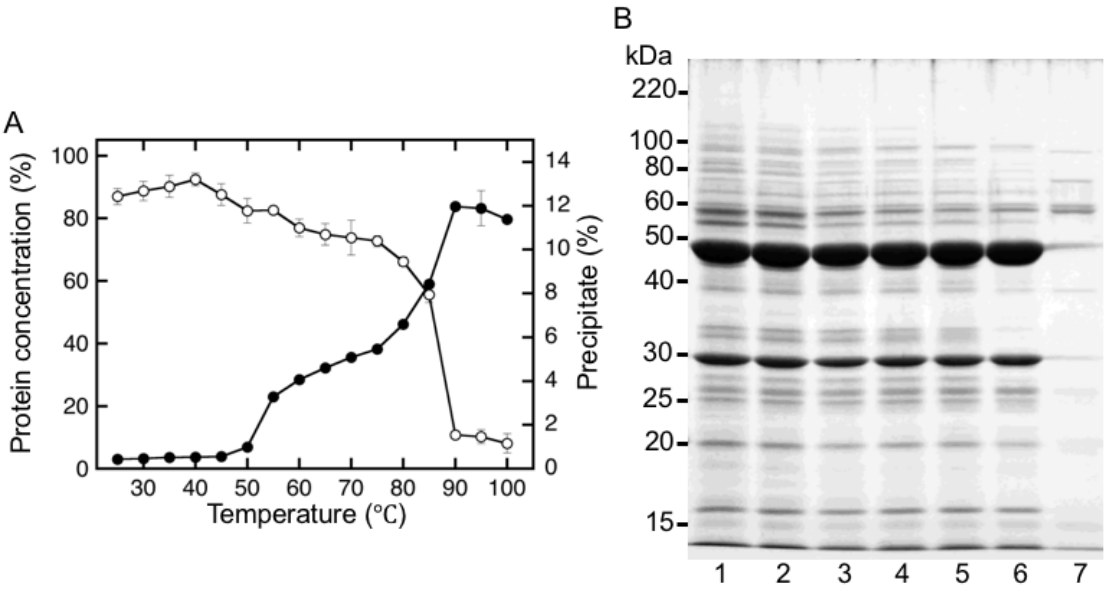

Figure 1.4 Temperature-dependent precipitate formation of sword bean proteins.
The effects of heating at various temperatures on the precipitation of sword bean proteins were analyzed. After heating, samples were separated into supernatants and precipitates. (A) The protein concentration of the supernatants (open circles) was determined using the Bradford method with bovine serum albumin as a standard. The ratio was calculated by dividing the concentration of the supernatant by that of the initial sample without heating and centrifugation. The weight of the wet precipitate was measured using a balancer. The precipitation efficiency was calculated by dividing the weight of the precipitate by that of the initial sample (closed circles). Data represent the average ± standard deviation of three independent experiments. (B) Sword bean proteins in Extract B (lane 1) were centrifuged (lane 2). The samples were heated at 25°C (lane 3), 50°C (lane 4), 55°C (lane 5), 75°C (lane 6), or 100°C (lane 7) and then centrifuged. The proteins in 12 μL reaction mixtures were subjected to SDS-PAGE (12.5% polyacrylamide) and stained with Coomassie Brilliant Blue R-250.

1.3.5. Magnesium chloride-dependent precipitate formation at various temperatures

During the production of tofu, soybean proteins are precipitated by adding $MgCl_2$ into soymilk at a concentration of approximately 20 mM (Arii and Takenata, 2013). Therefore, it is investigated whether adding $MgCl_2$ into Extract B at the same concentration (20 mM) would induce the precipitation of sword bean proteins (Figure 1.5). The protein concentration of Extract B was significantly decreased by the addition of $MgCl_2$ after heating at 25°C, 50°C, and 75°C (Figure 1.5A). The precipitation efficiency was significantly increased according to the decreasing protein concentrations at these temperatures (Figure 1.5B). The protein concentration and precipitation efficiency were similar for samples treated with or without $MgCl_2$ after heating at 100°C, although both the protein concentration and precipitation efficiency were slightly decreased at 100°C (Figure 1.5A and B).

Proteins in Extract B were also analyzed by SDS-PAGE (Figure 1.5C). Interestingly, the 48-kDa protein was clearly decreased in the supernatant following addition of $MgCl_2$ (Figure 1.5C, lanes 2, 4, and 6). These results suggested that addition of $MgCl_2$ induced precipitation of the 48-kDa protein from the crude extract. Analysis of the N-terminal amino acid sequence indicated that the protein consisted of the sequence His-Ser-Gly-His-Ser-Gly-Gly-Glu-Ala-Glu-, which was identical to the N-terminal sequence of sword bean canavalin matured through removal of the N-terminal signal sequence region comprising the amino acid residues from Met (Bressani *et al.*, 1987) to Ala (Yamauchi *et al*., 1988; Takei *et al*., 1989). In addition, the molecular weight of sword bean canavalin is estimated to be approximately 47.6 kDa from the primary sequence. Thus, these results suggested that sword bean canavalin was precipitated from the crude extract following addition of $MgCl_2$.

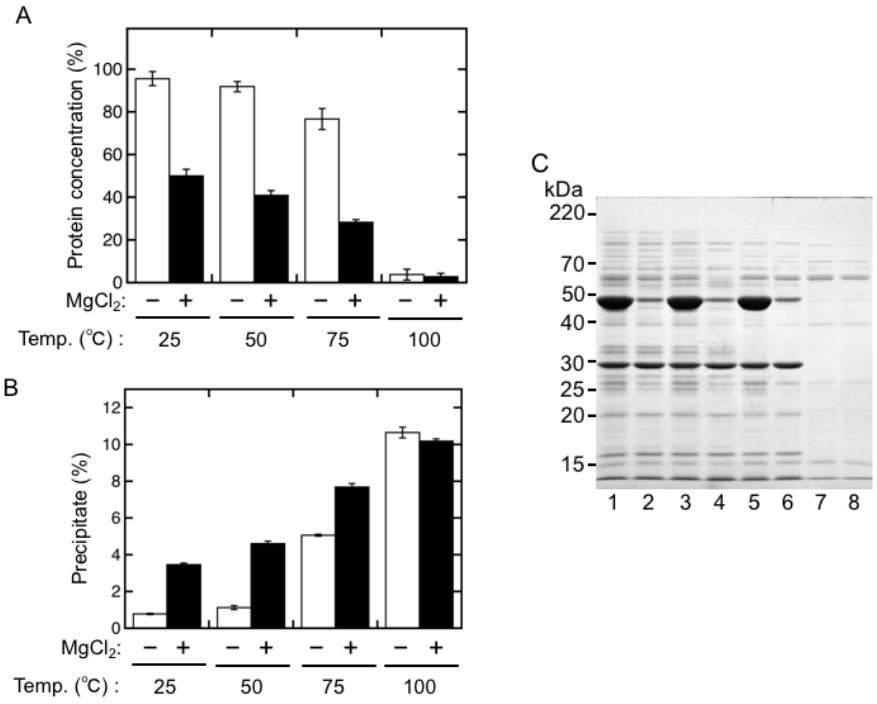

Figure 1.5 $MgCl_2$-dependent precipitation of sword bean proteins.

Samples were prepared by centrifugation of Extract B. Distilled water (white bars) or 200 mM $MgCl_2$ (black bars) was added to 9 volumes of sample, and samples were heated at 25°C, 50°C, 75°C, or 100°C. After heating at each temperature for 15 min, the mixtures were incubated on ice for 15 min and then separated into supernatants and precipitates by centrifugation. (A) The protein concentration of the supernatant was determined in the same way. The ratio was calculated by dividing the concentration of the supernatant by that of the initial sample plus distilled water without heating and centrifugation. Data represent the average ± standard deviation of three independent experiments. (B) The precipitation efficiency was calculated by dividing the weight of the precipitates by that of the initial sample. Data represent the average ± standard deviation of three independent experiments. (C) Distilled water (lanes 1, 3, 5, and 7) or 200 mM $MgCl_2$ (lanes 2, 4, 6, and 8) was added to 9 volumes of sample after heating at 25°C (lanes 1 and 2), 50°C (lanes 3 and 4), 75°C (lanes 5 and 6), or 100°C (lanes 7 and 8). After heating at each temperature for 15 min, the mixtures were incubated on ice for 15 min and then separated into supernatants and precipitates by centrifugation. Supernatant proteins in 12 μL reaction mixtures were subjected to SDS-PAGE (12.5% polyacrylamide) and stained with Coomassie Brilliant Blue R-250.

1.4. Discussion

In this study, I aimed to establish a novel method for the extraction of sword bean proteins from dried seeds in distilled water and to investigate the precipitation properties of sword bean proteins useful for food development. Interestingly, it is found that sword bean canavalin was specifically precipitated by addition of $MgCl_2$. To my knowledge, this is the first report showing that specific precipitation of sword bean canavalin was induced by addition of $MgCl_2$ to the crude extracts of sword bean. Thus, these data provide important insights into optimal methods for the extraction of proteins and for the production of processed foods from sword beans.

In previous studies, sword bean proteins have been extracted from ground whole seeds in a solution containing a high concentration of NaCl (0.15 mM) (Moreira and Cavada, 1984; Ceccatto *et al.*, 2002; Moreno *et al.*, 2004; Delaorre *et al.*, 2007; Bezerra *et al.*, 2007). However, protein extracts prepared with high concentrations of salts are not suitable for production of processed foods. Therefore, it is established a new method for the preparation of extracts from dried sword beans with distilled water, similar to the preparation of soymilk. Interestingly, it is found that the proteins were precipitated by heating at 100°C. This was in contrast to the preparation of soymilk, which is prepared by heating of raw soymilk at 100°C and has not been shown to cause precipitation of most soybean proteins (Ono *et al.*, 1991; Kohyama *et al.*, 1995; Guo *et al.*, 1997). Thus, most soybean proteins are likely to be more thermostable than sword proteins in heated extracts. Heating at less than 85°C permitted the preparation of extracts containing abundant proteins. In addition, substantial protein precipitation was observed by heating this extract containing abundant proteins at 100°C. As described in the Results section, it is obtained approximately 8.6 g wet precipitate from 100 g of extract. From these data, I estimated

that each dried seed might provide approximately 1.4 g of wet precipitate (with each dried bean weighting approximately 2.5 g, as shown in Table 1.1). In addition, heating at 55°C dramatically increased precipitate formation without a decrease in protein concentration. The inconsistency between the changes in precipitate formation and those in protein concentration implies that a non-proteinaceous polymer, such a polysaccharide, was precipitated by heating at 55°C. Thus, this substantial protein precipitation may provide the potential for the production of processed foods using sword beans as an alternative protein source; however, it should be noted that the wet precipitate may also contain various substances other than proteins.

Knowledge of the physicochemical properties of proteins within a bean product is important for the development of new processed foods, such as soybean products, as a superior protein source. During the production of tofu from soymilk, heating and the addition of $MgCl_2$ are important manipulations (Arii and Takenaka, 2013; Arii and Takenaka, 2014). In this study, heating at more than 90°C induced the precipitation of most of the sword bean proteins. In contrast, adding $MgCl_2$ induced the precipitation of only some sword bean proteins. From these results, I classified native sword bean proteins into three groups based on precipitate formation (Figure 1.6). As shown in Figure 1.6, proteins in the first group were not precipitated by heating at more than 90°C; proteins in the second group were precipitated by heating at more than 90°C but not by the addition of $MgCl_2$; and proteins in the third group were precipitated by both heating and the addition of $MgCl_2$. From SDS-PAGE analysis, it is found that this third protein group was comprised primarily of a single protein. Further analysis allowed us to identify this protein as canavalin based on the N-terminal amino acid sequence and molecular weight estimation. Thus, the precipitated proteins shown by black circles in Figure 1.6 were

mainly canavalin.

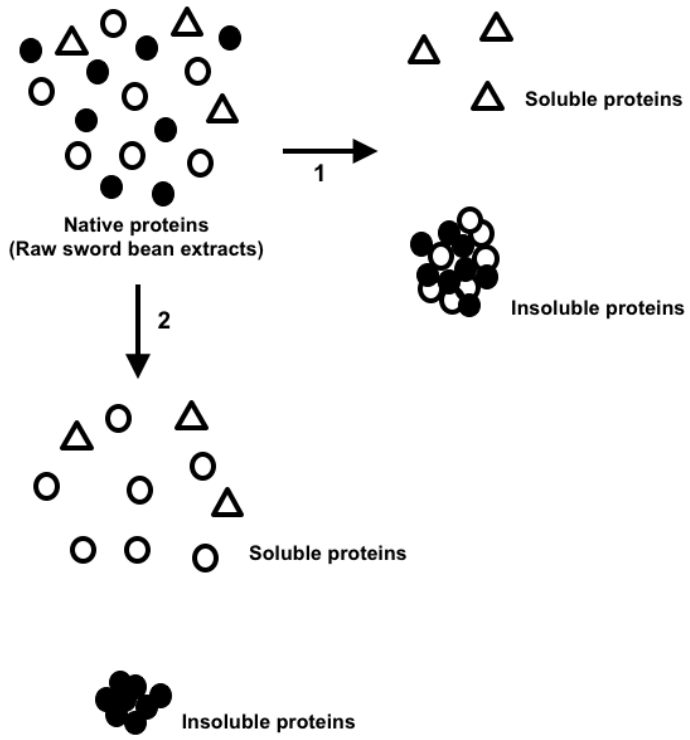

Figure 1.6 Schematic model for the precipitation of sword bean proteins under different conditions.

Circles show proteins precipitated by heating at a high temperature (> 90°C). Triangles show proteins soluble at the high temperature. Open symbols show proteins that were not precipitated by adding $MgCl_2$. Closed symbols show proteins that were precipitated by adding $MgCl_2$. Arrows 1 and 2 indicate heating at more than 90°C and the addition of 20 mM $MgCl_2$ at temperatures ranging from 25°C to 75°C, respectively.

Canavalin was first isolated from jack beans (*Canavalia ensiformis*) (Sumner and Howel, 1919) and is classified as vicilin or 7S type based on the primary structure (Gibbs *et al.*, 1989). The primary structure of jack bean canavalin has been determined using the cDNA sequence (Ng *et al.*, 1992; Ng *et al.*, 1993). The primary structure of sword bean canavalin has also been determined using the cDNA sequence (Yamauchi *et al.*, 1988; Takei *et al.*, 1989). As described above, the N-terminal sequence of the protein precipitated by the addition of $MgCl_2$ was completely identical to the N-terminal sequence of sword bean canavalin matured by the removal of the signal sequence region. Only two of the 419 amino acid residues in the mature form differ between the primary sequences of sword bean canavalin and jack bean canavalin. From the high homology of the primary structures, I can assume the functions and structures of the proteins are highly similar. Canavalin is a major storage protein; however, the physiological function of canavalin is still unclear. It is found that sword bean canavalin was precipitated following addition of $MgCl_2$. No previous studies have reported precipitation of sword bean or jack bean canavalin induced by the addition of $MgCl_2$. Interestingly, several studies have characterized the mechanisms through which $MgCl_2$ induces protein precipitation. For example, during tofu formation, the addition of divalent cations functions as an initiating factor and facilitates direct interaction among soybean proteins to induce protein precipitation (Arii and Takenaka, 2014). The primary and tertiary structures of canavalin highly resemble those of soybean β-conglycinin, which is a soybean 7S globulin (Maruyama *et al.*, 2001). Soybean 7S globulin is also precipitated by the addition of divalent cations (Appu and Narasinga, 1976). Although the primary and tertiary structures of adzuki 7S globulins and common bean phaseolin also highly resemble those of canavalin (Maruyama *et al.*, 2001; Fukuda *et al.*, 2008), to my knowledge, these proteins

have not been reported to be precipitated by $MgCl_2$. It is also unclear whether similar precipitation is induced for bean proteins other than soybean and sword bean proteins. These features provide an important insight into the production of processed foods, not only for sword beans but also for other beans. The characteristics of other bean proteins should thus be addressed in future studies. In addition, annexin IV protein interacts with a non-proteinaceous substance through divalent cations coordinated to proteins (Fukuda *et al.*, 2008). Consistent with this, it is also found evidence of non-proteinaceous substances in this extract. I would expect that canavalin interacts with such non-proteinaceous substances, facilitating precipitation in the presence of magnesium ions. Thus, I will examine the molecular mechanisms of canavalin precipitation in future studies. These data clearly support the hypothesis that sword bean canavalin may have applications as a new food material for the formation of gels and curds. In addition, analysis of the physicochemical characteristics of sword bean proteins has led to the discovery of a new physiological function of canavalin.

Canavalia seeds contain several antinutritional compounds, such as total tannins, trypsin inhibitors, chymotrypsin inhibitors, phytic acid, canavanine, α-amylase inhibitors, lectins, and total saponins (Siddhurahu and Becker, 2001; Arii *et al.*, 2015; Makkar *et al.*, 2007). In animal experiments, sufficient soaking and heating are effective for reducing the amounts of these antinutritional compounds (Sasipriya and Siddhuraju, 2013; Makkar *et al.*, 2007; Belitz *et al.*, 2009). Although sword beans are traditionally eaten (Siddhuraju and Becker, 2001; Bezerra *et al.*, 2007), more studies will be needed to determine the suitability of sword beans as a food material in the production of processed foods. Interestingly, consumption of sword beans and sword bean extracts has been reported to prevent bone loss (Byun and Lee, 2010; Nakatsuka *et al.*, 2014). Although it is unclear

whether or not some sword bean proteins prevent bone loss, it is likely that humans can derive functional benefits by eating processed foods containing sword beans. Therefore, sword beans remain an attractive food material for use in the production of processed foods and may have applications as a functional food with health benefits.

In the present study, consistent with the preparation of soymilk extract, I found that soaking time was an important factor determining water absorption in dried sword beans. Maximum water absorption was obtained after more than 16 h of soaking, as measured by changes in bean weight and size. For the preparation of extracts in this study, beans were soaked in distilled water for 18 h. However, it is unclear whether this long soaking time would remove the antinutritional compounds to levels below safety thresholds. Previous studies have shown that shorter soaking times (12 and 15 h) are sufficient for the safe preparation of sword beans (Sasipriya and Siddhuraju, 2013; Ekanayake *et al*., 2007). Thus, for the safe use of sword beans, long soaking times and repeated water exchanges may be needed.

Interestingly, the changes in the sizes of soybeans were different from the trends observed for sword beans during soaking. This difference may result from differences between the shapes of the dried beans and those of the soaked beans. For example, for soybeans, the three-dimensional shape of a dried bean is almost a complete sphere, while that of a soaked bean resembles a bean-shaped sphere. In contrast, sword beans resemble bean-shaped spheres both as dried beans and as soaked beans, with a uniform increase in size during soaking.

This extract containing abundant proteins is prepared without heating. Sword bean has three trypsin inhibitors, which are the same Bowman-Birk proteinase inhibitors family with the soybean inhibitor (Park *et al.*, 2000). In contrast, when the raw extract is

heated at 100°C, most sword bean proteins are precipitated. The heated extract cannot be used as an alternative protein source. These evidences indicate that it is difficult to use the sword bean extract as a drinkable food product such as soymilk. However, the protein precipitates by only heating would be rightly utilized for the production of processed foods as an alternative protein source. In addition, the canavalin precipitate induced by adding $MgCl_2$ would also be utilized for the production of processed foods by the following heating to the precipitation.

In conclusion, I proposed a new method for the preparation of extracts from dried sword beans using distilled water, which is similar to the preparation of soymilk. Heating and the addition of $MgCl_2$ induced precipitation of sword bean proteins from the crude extract. In addition, native sword bean proteins were classified into three groups based on precipitate formation, and I observed precipitation of canavalin protein from the crude extracts following addition of $MgCl_2$. Thus, these data provided important information regarding the physicochemical properties of sword beans that may be useful for the production of processed foods.

Chapter 2

Reversible changes of canavalin solubility controlled by divalent cation concentration in crude sword bean extract

2.1. Introduction

White sword bean (*Canavalia gladiata*) is a part of the traditional diet in the Asian tropics and subtropics (Bressani *et al.*, 1987; Siddhuraju and Becker, 2001) and is relatively resistant to pests and most diseases (Smartt, 1976). Since the dried beans contain approximately 25% crude proteins (Bressani *et al.*, 1987; Sasipriya and Siddhuraju, 2013), they are good potential sources of protein. In Chapter 1, I have reported a method for the extraction of some proteins from sword bean using only distilled water. In addition, the predominant protein in the crude extract was determined to be canavalin.

Canavalin is the major storage protein of sword bean and jack bean (*Canavalia ensiformis*) with a molecular mass of 47.6 kDa, classified as 7S seed globulin or legume vicilin (Sumner *et al.*, 1983). It was first isolated from jack bean (Sumner and Howel, 1919), and later its primary structure was determined in both bean species using cDNA sequence (Ng *et al.*, 1992; Ng *et al.*, 1993; Yamauchi *et al.*, 1988; Takei *et al.*, 1989). These two primary structures differ in only two out of 419 amino acids of canavalin (Maruyama *et al.*, 2001; Fukuda *et al.*, 2008) and have high homology with other legume 7S globulins (Maruyama *et al.*, 2001; Fukuda *et al.*, 2008; Sammour *et al*., 1984; Gibbs *et al*., 1989), which probably shows that they may also similar tertiary structure, physiological function, and physicochemical properties. Although the tertiary structure of sword bean canavalin has not been yet determined, several X-ray crystal structure analyses showed that jack bean canavalin is a homotrimer (Ko *et al.*, 1993a; Ko *et al.*,

1993b; Ko *et al.*, 2000; Ko *et al.*, 2001; McPherson, 1980). The tertiary structure highly resembles that of soybean β-conglycinin, a soybean 7S globulin (Maruyama *et al.*, 2001). Soybean 7S globulin is precipitated by the addition of divalent cations (Appu and Narasinga, 1976). This characteristic of soybean 7S globulin is utilized for the processing of tofu. If canavalin were also to possess similar characteristics, this protein could be useful for the manufacture of processed foods.

In Chapter 1, it is reported a method for the extraction of proteins from sword bean in distilled water and their precipitation by the addition of 20 mM $MgCl_2$ in the crude extract. This was the first study that reported the precipitation of canavalin in the presence of $MgCl_2$ and showed that a detailed investigation of canavalin precipitation might be important for the study of its physiological function and physicochemical properties.

In this study, we examined the effects of the addition of $MgCl_2$, $CaCl_2$, and NaCl to the crude sword bean extract at various concentrations on the solubility of canavalin. These findings might provide useful information for the study of the physiological functions and physicochemical properties of canavalin and its application in food industry.

2.2. Materials and methods

2.2.1. Materials

White sword beans were purchased from Morika Beiten (Nara, Japan) and general chemical reagents from Wako Pure Chemical Industries (Osaka, Japan).

2.2.2. Preparation of sword bean extract

A sword bean extract was prepared as described in Chapter 1. Briefly, soaked sword beans

were ground for 5 min in eight volumes (v/w) of distilled water using a hand blender (CSB-77JBSTRW; Cuisinart, Stamford, CT) on ice, and the suspension was sieved through a cotton cloth. The extract was centrifuged at 9,100 × *g* for 10 min at 4°C, and the supernatant was used for analysis.

2.2.3. SDS-PAGE

Samples were mixed with 0.33 volumes of SDS (0.25 M Tris-HCl at pH 7.0, containing 4% SDS, 5% 2-mercaptoethanol, and 40% glycerol) and incubated in a water bath at 100°C for 5 min. SDS-PAGE was conducted using sample volumes corresponding to 0.25 µl of the sword bean extract; this volume contained approximately 4.0 µg proteins in the absence of divalent cations (Chapter 1). SDS-PAGE was carried out on 10% polyacrylamide gels at a constant current of 12.5 mA for 2.5 h as described previously (Laemmli, 1970). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250. The molecular weight standard was purchased from Life Technologies (Tokyo, Japan).

2.2.4. Analysis of supernatant proteins

The sword bean extract was incubated at 25°C for 5 min. Next, 0.11 volumes of $MgCl_2$, $CaCl_2$, NaCl, or distilled water (control) were added to the incubated sample at various concentrations, mixed, and incubated at 25°C for 15 min and on ice for 5 min. The mixtures were centrifuged at 9,100 × *g* for 20 min at 4°C. The supernatant was diluted 15-fold with distilled water. The supernatant proteins were analyzed by 10% SDS-PAGE.

2.2.5. Determination of residual canavalin ratio

The intensity of canavalin bands on SDS-polyacrylamide gel was quantified by ImageJ

(National Institutes of Health, Bethesda, MD) (Abràmoff *et al.*, 2004). The residual canavalin ratio was expressed as the percentage of band intensity of the sample with added metal chloride to that of the sample with distilled water. Data were expressed as mean ± standard deviation of three independent experiments.

2.2.6. Analysis of insoluble canavalin solubilization

Insoluble canavalin was prepared by the addition of 15 mM $MgCl_2$ or 10 mM $CaCl_2$ to sword bean extract. Canavalin precipitated in the former was suspended in 60 mM $MgCl_2$ or distilled water, whereas that precipitated in the latter was suspended in 200 mM $CaCl_2$ or distilled water. The mixtures were centrifuged at 9,100 × *g* for 20 min at 4°C. The supernatant was diluted 15-fold with distilled water to adjust the concentration of divalent cations to less than 15 mM. The precipitates were dissolved in a volume of 8 M urea equal to that of the initial extract. Proteins were analyzed using 10% SDS-PAGE.

2.2.7. Analysis of soluble canavalin insolubilization

Soluble canavalin was prepared by the addition of 60 mM $MgCl_2$ or 200 mM $CaCl_2$ to sword bean extract. The solutions were diluted 4-fold or 20-fold, respectively, with distilled water. The diluted mixtures were centrifuged at 9,100 × *g* for 20 min at 4°C. The $MgCl_2$ supernatant was diluted 3-fold with distilled water such that the $MgCl_2$ concentration was 5 mM. The $CaCl_2$ concentration of the $CaCl_2$ supernatant is adjusted to 10 mM. The precipitates were prepared as described for the analysis of insoluble canavalin solubilization. Proteins were analyzed using 10% SDS-PAGE.

2.2.8. Curve fitting analysis

The residual canavalin ratio was plotted against each metal chloride concentration. The plots were fitted to the following equation 2.1 using the KaleidaGraph 4.5 software (Synergy Software, PA, USA):

$$r = r_{max} + (r_{min} - r_{max}) / (1 + [C] / C_m)^d \quad (2.1)$$

where r is the residual canavalin ratio at a metal chloride concentration C, r_{max} is the maximum ratio, r_{min} is the minimum ratio, C_m is the midpoint metal chloride concentration, and d is the slope coefficient at the midpoint (Arii and Takenaka, 2014; Tang *et al.*, 2012). Data represent the average ± standard deviation of five independent experiments.

2.3. Results

2.3.1. Effects of $MgCl_2$ and NaCl on canavalin solubility

I first investigated the $MgCl_2$ and NaCl concentration-dependency of canavalin precipitation (Figure 2.1). The results showed that the minimum ratio of residual canavalin was 7.1 ± 3.5% at 15 mM $MgCl_2$, whereas the maximum was 78.4 ± 5.6% at 50 mM $MgCl_2$. Therefore, canavalin was insolubilized at relatively low concentrations of $MgCl_2$ (< 20 mM) and solubilized at relatively high concentrations (> 20 mM). When NaCl was added into the crude extract, canavalin solubilization was slightly decreased, and the minimum ratio of residual canavalin was 84.9 ± 3.3% at 25 mM NaCl. To investigate whether canavalin solubilization was highly decreased at NaCl concentrations of more than 50 mM, I also studied it at the range of 100–400 mM (Figure 2.2). The

results showed that at 100–350 mM, approximately 75% of canavalin remained in the supernatant (Figure 2.2B). The minimum ratio of residual canavalin was 75.1 ± 1.2% at 200 mM NaCl, whereas the maximum was 92.2 ± 5.3% at 400 mM NaCl. Therefore, although canavalin was soluble in the presence of Na^{+}, Mg^{2+} had a more significant effect on canavalin solubility.

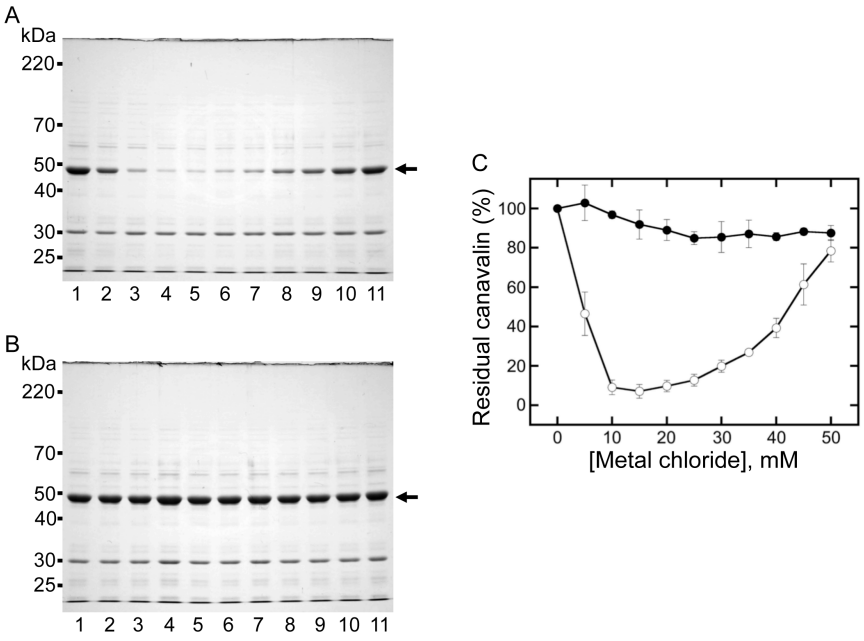

Figure 2.1 Effects of $MgCl_2$ and NaCl concentrations on canavalin solubility.

$MgCl_2$ (A) and NaCl (B) were added to sword bean extract at concentrations of 0–50 mM. $MgCl_2$ and NaCl were added to the sword bean extract at a concentration of 5 mM (lane 2), 10 mM (lane 3), 15 mM (lane 4), 20 mM (lane 5), 25 mM (lane 6), 30 mM (lane 7), 35 mM (lane 8), 40 mM (lane 9), 45 mM (lane 10), and 50 mM (lane11). Distilled water was used instead of $MgCl_2$ as a control (lane 1). The mixture was centrifuged, and the supernatant was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The arrows show canavalin bands. The proportion of residual canavalin in the supernatant was estimated from band intensity using ImageJ (C). Open and closed circles indicate $MgCl_2$ and NaCl, respectively. Data were expressed as mean ± standard deviation of three independent experiments.

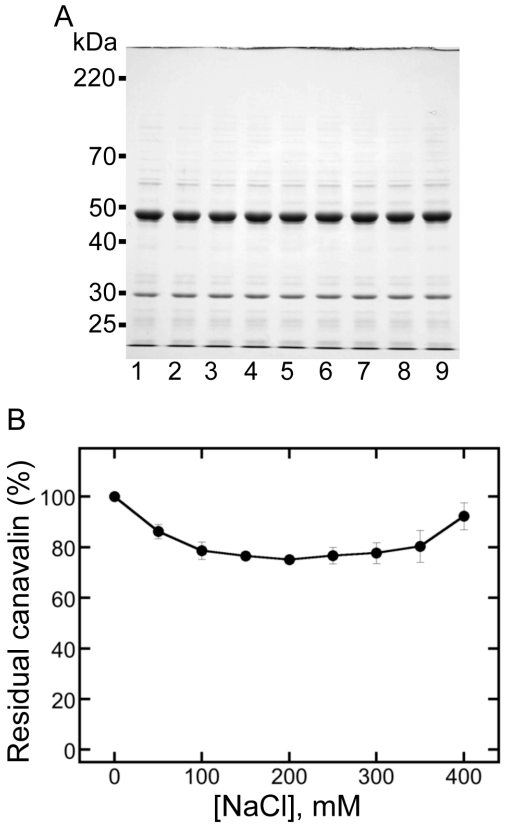

Figure 2.2 Effects of high NaCl concentrations on canavalin solubility.

NaCl was added to the sword bean extract at a concentration of 0–400 mM. NaCl was added to the sword bean extract at a concentration of 50 mM (lane 2), 100 mM (lane 3), 150 mM (lane 4), 200 mM (lane 5), 250 mM (lane 6), 300 mM (lane 7), 350 mM (lane 8), and 400 mM (lane 9). Distilled water was used instead of $MgCl_2$ as a control (lane 1). Proteins were analyzed (A) and quantified (B) as described in Figure 2.1. Data were expressed as mean ± standard deviation of three independent experiments.

2.3.2. Changes in canavalin solubility induced by $MgCl_2$

Canavalin reaction to $MgCl_2$ was divided into the insolubilization phase in the range of 0–20 mM $MgCl_2$ and the solubilization phase in the range of 20–50 mM $MgCl_2$ (Figure 2.1C). Therefore, the effect of $MgCl_2$ on canavalin solubility was investigated separately in each phase (Figure 2.3). In the range of 0–20 mM $MgCl_2$, soluble canavalin was highly decreased with the increasing concentration of $MgCl_2$ (Figure 2.3A). The reduction of

canavalin in the supernatant was more than 90% (Figure 2.3B) at concentrations higher than 12 mM $MgCl_2$, reaching a maximum reduction at 16 mM $MgCl_2$ (5.9 ± 2.3%). In the range of 20–60 mM, supernatant canavalin was sigmoidally increased with the increasing concentration of $MgCl_2$, reaching a plateau at 50 mM $MgCl_2$ (Figure 2.3C and D). The highest amount of supernatant canavalin was 91.2 ± 7.0% at 55 mM $MgCl_2$. These results showed that canavalin tends to be insolubilized at relatively low concentrations of $MgCl_2$ (< 20 mM) and solubilized at relatively high concentrations (> 20 mM).

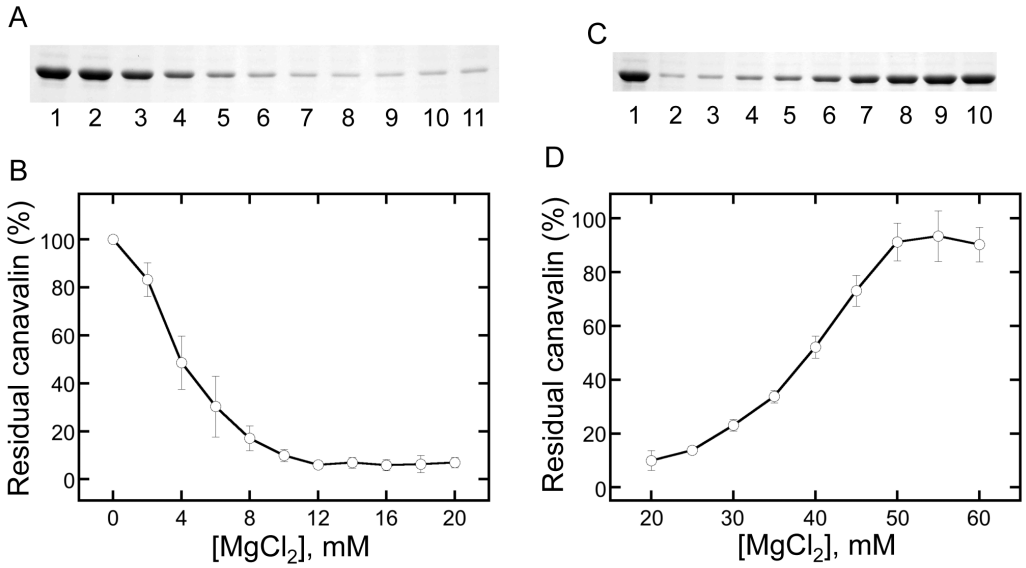

Figure 2.3 Analysis of canavalin solubility induced by $MgCl_2$.

The decreasing (A and B) and increasing (C and D) intensity of canavalin band was investigated as described in Fig. 1. (A) $MgCl_2$ was added to the sword bean extract at a concentration of 2 mM (lane 2), 4 mM (lane 3), 6 mM (lane 4), 8 mM (lane 5), 10 mM (lane 6), 12 mM (lane 7), 14 mM (lane 8), 16 mM (lane 9), 18 mM (lane 10), and 20 mM (lane11). Distilled water was used instead of $MgCl_2$ as a control (lane 1). (B) $MgCl_2$ was added to the sword bean extract at a concentration of 20 mM (lane 2), 25 mM (lane 3), 30 mM (lane 4), 35 mM (lane 5), 40 mM (lane 6), 45 mM (lane 7), 50 mM (lane 8), 55 mM (lane 9), and 60 mM (lane 10). Distilled water was used instead of $MgCl_2$ as a control (lane 1). Proteins were quantified as described in Figure 2.1 (B and D). Data were expressed as mean ± standard deviation of three independent experiments.

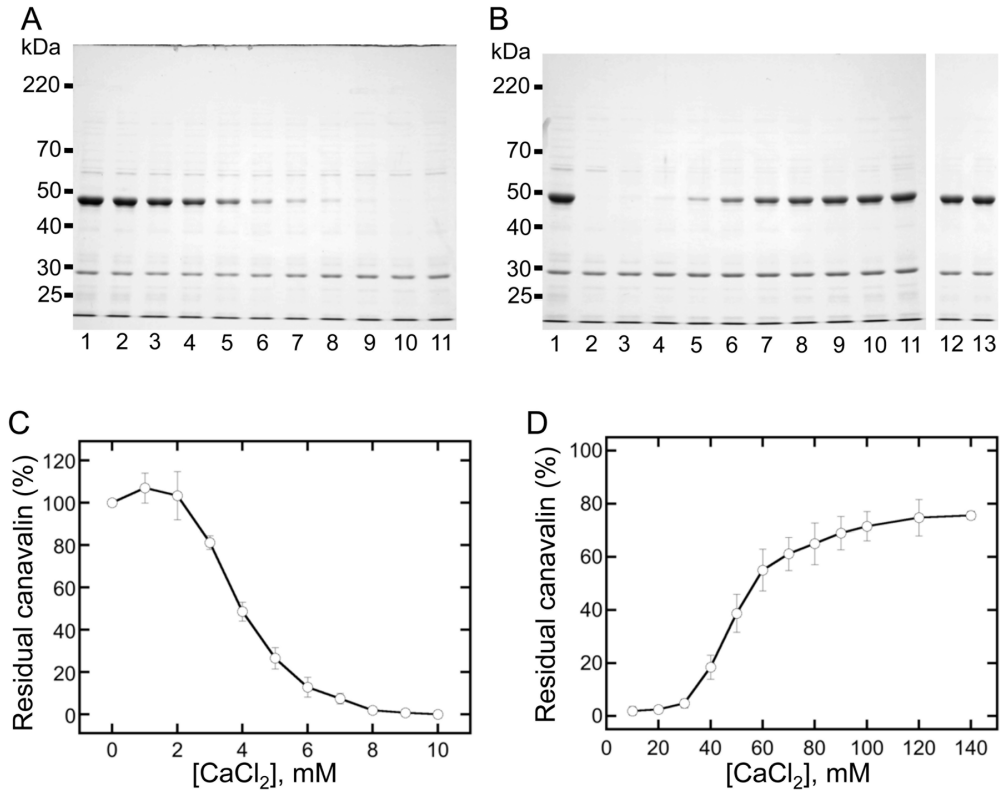

Figure 2.4 Effects of $CaCl_2$ concentration on canavalin solubility.

$CaCl_2$ was added to the sword bean extract at a concentration of 0–10 mM (A and C) and 10–140 mM (B and D). Proteins were analyzed (A and B) and quantified (C and D) as described in Figure 2.1. (A) $CaCl_2$ was added to the sword bean extract at a concentration of 1 mM (lane 2), 2 mM (lane 3), 3 mM (lane 4), 4 mM (lane 5), 5 mM (lane 6), 6 mM (lane 7), 7 mM (lane 8), 8 mM (lane 9), 9 mM (lane 10), and 10 mM (lane11). Distilled water was used instead of $CaCl_2$ as a control (lane 1). (B) $CaCl_2$ was added to the sword bean extract at a concentration of 10 mM (lane 2), 20 mM (lane 3), 30 mM (lane 4), 40 mM (lane 5), 50 mM (lane 6), 60 mM (lane 7), 70 mM (lane 8), 80 mM (lane 9), 90 mM (lane 10) 100 mM (lane11), 120 mM (lane 12), and 140 mM (lane 13). Distilled water was used instead of $CaCl_2$ as a control (lane 1). Data were expressed as mean ± standard deviation of three independent experiments.

2.3.3. Effect of $CaCl_2$ on canavalin solubility

To investigate the specificity of divalent cations, I also examined the effect of $CaCl_2$ on the solubility of canavalin (Figure 2.4). Canavalin reaction to $CaCl_2$ was divided into the

insolubilization phase in the range of 0–10 mM $CaCl_2$ (Figure 2.4A and C) and the solubilization phase in the range of 20–60 mM $CaCl_2$ (Figure 2.4B and D). Canavalin completely disappeared in the range of 9–10 mM (Figure 2.4C); it was sigmoidally increased by approximately 75% in the range of 10–140 mM (Figure 2.4D); whereas 75.1 ± 1.2% was solubilized at 200 mM (Data not shown). These results indicated that $CaCl_2$ played a similar role with that of $MgCl_2$ and that the concentration of divalent cations significantly affects the solubility of canavalin.

2.3.4. Comparison between effects of Mg^{2+} and Ca^{2+} on canavalin solubility

To compare the effect of Mg^{2+} and Ca^{2+} on canavalin solubility, the midpoint concentrations (C_m) in the insolubilization and solubilization phases were determined by curve fitting analysis (Figures 2.3B and D, 2.4C and D). As summarized in Table 2.1, the C_m value of $MgCl_2$ in the insolubilization phase was similar to that of $CaCl_2$. However, $MgCl_2$ induced the insolubilization of canavalin at 2 mM (Figure 2.3B), whereas $CaCl_2$ at the same concentration did not (Figure 2.4C). The C_m value of $MgCl_2$ in the solubilization phase was lower than that of $CaCl_2$ (Table 2.1). In addition, canavalin was almost completely recovered by the addition of $MgCl_2$ (Figure 2.3D), but only incompletely by the addition of $CaCl_2$ (Figure 2.4D). Overall, the results suggested that the effect of Mg^{2+} on the solubilization or insolubilization of canavalin was stronger than that of Ca^{2+}.

Table 2.1 Midpoint concentrations for the insolbulization and solubilization of canavalin.

	Insolubilization		Solubilization	
	C_m[a] (mM)	R^2[b]	C_m[a] (mM)	R^2[b]
$MgCl_2$	3.9	0.998	40.0	0.992
$CaCl_2$	3.9	0.998	50.0	0.996

[a]C_m indicates the midpoint concentration. The values were determined by the curve fitting analysis using the command for the sigmoidal equation of a graphic soft, KaleidaGraph 4.6.
[b]R^2 indicates the coefficient of determination for the curve fitting analysis and was estimated using the software.

2.3.5. Reversibility of canavalin solubility

I examined whether insolubilized canavalin was solubilized in distilled water or in the presence of $MgCl_2$ and $CaCl_2$ (Figure 2.5). Canavalin was first insolubilized by the addition of 15 mM $MgCl_2$ (Figure 2.5A, lanes 2 and 3), and most canavalin was observed in the precipitate fraction (Figure 2.5A, lane 3). The precipitated proteins were suspended in distilled water (Figure 2.5A, lanes 4 and 5), and only a small amount was dissolved. In contrast, the highest amount of precipitated proteins was dissolved in 60 mM $MgCl_2$ (Figure 2.5A, lanes 6 and 7), and then, the insolubilization of resolubilized proteins was increased with decreasing concentrations of $MgCl_2$ from 60 mM to 15 mM (Figure 2.5A, lanes 8 and 9).

Canavalin was first insolubilized by the addition of 10 mM $CaCl_2$ (Figure 2.5B, lanes 2 and 3). The precipitated proteins were suspended in distilled water (Figure 2.5B, lanes 4 and 5), and only a small amount was dissolved. The precipitated proteins were also suspended in 200 mM $CaCl_2$ (Figure 2.5B, lanes 6 and 7), in which the highest amount was dissolved. The insolubilization of resolubilized proteins was increased with the decreasing concentration of $CaCl_2$ from 200 mM to 10 mM (Figure 2.5B, lanes 8 and

9). These results indicated that $CaCl_2$ also induces reversible solubility changes in canavalin.

It was also examined whether canavalin solubilized in the presence of Mg^{2+} and Ca^{2+} at relatively high concentrations was precipitated by decreasing the concentration of $MgCl_2$ or $CaCl_2$, respectively (Figure 2.6). Canavalin was solubilized by the addition of 60 mM $MgCl_2$ (Figure 2.6A) or 200 mM $CaCl_2$ (Figure 2.6B), and the soluble proteins were precipitated with the decreasing concentration of $MgCl_2$ (Figure 2.6A, lane 5) or $CaCl_2$ (Figure 2.6B, lane 5), respectively. The results indicated that canavalin was reversibly solubilized and insolubilized by the changing concentrations of Mg^{2+} and Ca^{2+}.

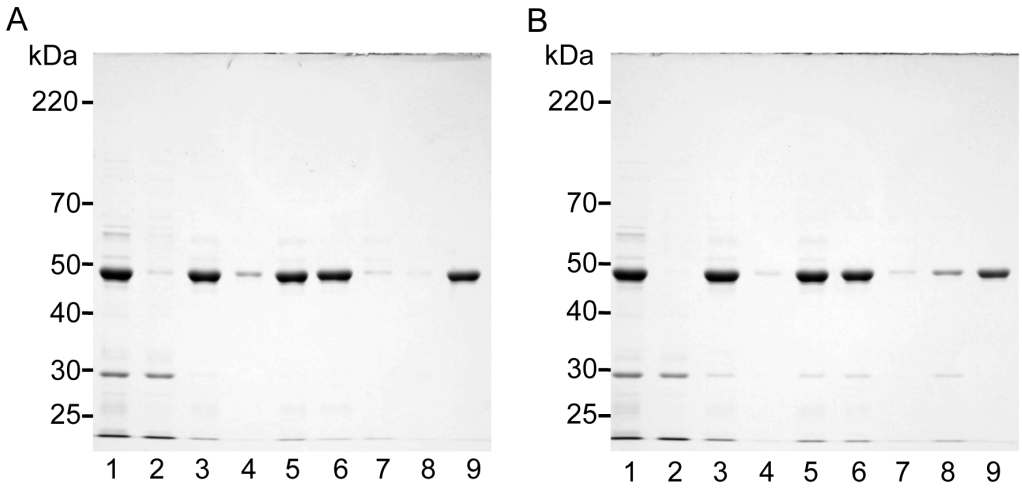

Figure 2.5 Solubility changes to insolubilized canavalin.

$MgCl_2$ (A) and $CaCl_2$ (B) were added to the sword bean extract at a concentration of 15 mM and 10 mM, respectively. Distilled water was used instead of salt as a control (lane 1). The mixture was separated into the supernatant (lane 2) and precipitate (lane 3). The precipitate was suspended in the same volume of distilled water, and then separated into the supernatant (lane 4) and precipitate (lane 5). The precipitate was also suspended in 60 mM $MgCl_2$ or 200 mM $CaCl_2$. The suspension was separated into the supernatant (lane 6) and precipitate (lane 7). The supernatant was diluted 4-fold or 20-fold, respectively. The diluted solution was separated into the supernatant (lane 8) and precipitate (lane 9). Proteins in the precipitate were dissolved in 8 M urea and subjected to 10% SDS-PAGE.

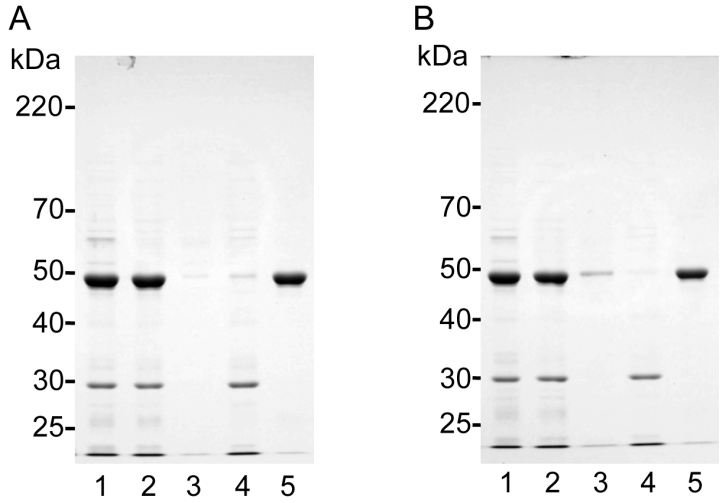

Figure 2.6 Solubility changes to solubilized canavalin.

$MgCl_2$ (A) and $CaCl_2$ (B) were added to the sword bean extract at a concentration of 60 mM and 200 mM, respectively. Distilled water was used instead of salt as a control (lane 1). The mixture was separated into the supernatant (lane 2) and precipitate (lane 3). The supernatant was diluted 4-fold or 20-fold, respectively. The diluted solution was separated into the supernatant (lane 4) and precipitate (lane 5). Proteins in the precipitate were dissolved in 8 M urea and subjected to 10% SDS-PAGE.

2.4. Discussion

In Chapter 1, it is indicated that canavalin is precipitated by the addition of 20 mM $MgCl_2$, whereas in the present study, it is showed that canavalin was insolubilized in the presence of divalent cations at relatively low concentrations and solubilized in the presence of the same cations at relatively high concentrations. In addition, I formulated a schematic representation of the reversible induction of canavalin insolubilization and solubilization by changing concentrations of Mg^{2+} and Ca^{2+} (Figure 2.7). Soluble canavalin was prepared as described in Chapter 1 and precipitated by the addition of $MgCl_2$ or $CaCl_2$ at relatively low concentrations, but not by the addition of NaCl (arrow 1). The insolubilized canavalin was not resolubilized in distilled water (arrow 2), but only in the presence of

Mg^{2+} and Ca^{2+} at relatively high concentrations (arrow 3). In addition, the soluble canavalin was not precipitated by the addition of 60 mM Mg^{2+} or 200 mM Ca^{2+} (arrow 5), but it was with the decreasing concentration of Mg^{2+} and Ca^{2+} (arrow 4). Based on these results, I assumed that the soluble canavalin prepared by the addition of relatively high concentration of divalent cations to the crude extract was the same with that prepared by the addition of relatively high concentration of divalent cations to insoluble canavalin.

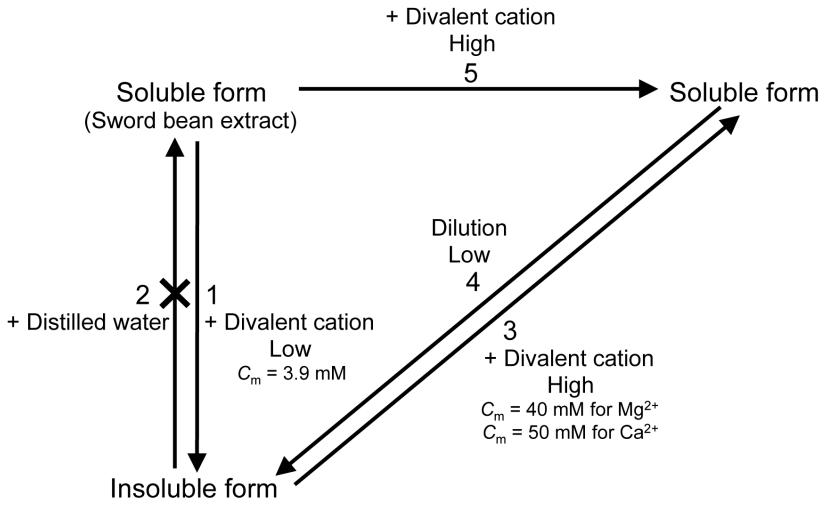

Figure 2.7 Schematic representation of canavalin solubility.

Arrows show changes in the concentration of divalent cations. Cross mark indicates no progress. 'Low' indicates 15 mM Mg^{2+} or 10 mM Ca^{2+}. 'High' indicates 60 mM Mg^{2+} or 200 mM Ca^{2+}.

In the solubilization phase, the C_m value was determined at 40 mM for Mg^{2+} and at 50 mM for Ca^{2+}. The results clearly indicated that the insolubilized canavalin was more easily solubilized in a solution containing Mg^{2+} than in a solution containing Ca^{2+}. In the insolubilization phase, the C_m value was determined at 3.86 mM for Mg^{2+} and 3.93 mM for Ca^{2+}. The similar C_m values indicated that the effects of Mg^{2+} and Ca^{2+} on canavalin insolubilization do not differ significantly. However, the sigmoidal graphs of canavalin insolubilization (Figures 2.3B and 2.4C) suggested that the protein shows a higher sensitivity to Mg^{2+} than to Ca^{2+} from the different initial concentrations for the insolubilization. A previous study indicated that different metal ion species influence the C_m value of protein association during tofu formation (Arii and Takenaka, 2014). The C_m value is significantly negatively correlated with the stability constant of EDTA for metal ions, and this strong correlation influences the interaction between metal ions and the carboxyl groups of soymilk proteins. Studies on the primary structure of canavalin (Yamauchi *et al.*, 1988; Takei *et al.*, 1989) indicate that the 419 amino acid residues of the matured form of canavalin include 36 glutamic acid residues and 23 aspartic acid residues. Therefore, changes in canavalin solubility might be also induced by the interaction between metal ions and the carboxyl groups of the protein. However, it was difficult to clarify the mechanism underlying the modulation of solubility change, because the sword bean extract contained many non-proteinaceous substances, such as polysaccharides, that might also interact with canavalin. Thus, further studies are needed to reveal the underlying molecular mechanism.

Sword bean contains abundant calcium and magnesium ions (Ekanayake *et al.*, 1999). Based on studies by Ekanayake *et al.* (1999), I estimated the Ca^{2+} and Mg^{2+} concentrations in sword bean extract to be approximately 4.6 mM and 8.8 mM,

respectively. The divalent cations inherently present in sword bean affect the primary solubility change of canavalin. Thus, these results must be interpreted with consideration of the fact that the C_m values in this study indicate the midpoint concentration for the insolubilization and solubilization in the sword bean extract prepared by the method I describe, but might not represent the essential features of canavalin. At present, since highly pure canavalin is insoluble in distilled water (Figure 2.5), it is experimentally difficult to determine the C_m value of purified canavalin for the divalent cations. However, it would be important to determine the C_m value of the unrefined canavalin in the extract to facilitate the use of canavalin in food processing. For example, tofu is also processed by the addition of divalent cations to extracts of soybean, which also contains abundant divalent cations. As I have previously reported in Chapter 1, the methodologies used in tofu production could be applied to the development of processed foods using canavalin. In previous study, I determined C_m values for tofu formation, and a comparison of C_m values between various metal ions clarified the role of metal ions in tofu formation (Arii and Takenaka, 2014). The C_m value for canavalin precipitation would thus be useful information for the development of processed foods using canavalin.

Some proteins are precipitated by the addition of salts, whereas others are solubilized. For example, during tofu formation, soybean proteins are precipitated by an increase in divalent cations (Arii and Takenaka, 2013; Arii and Takenaka, 2014), while chymosin B, β-lactoglobulin B and pumpkin seed globulin demonstrate salting-in behavior—that is, they are solubilized in the presence of high salt concentrations (Maurer *et al.*, 2011). In the present study, canavalin was insolubilized by the addition of divalent cations at relatively low concentrations, but was solubilized by the addition of the same cations at relatively high concentrations. Briefly, canavalin solubility can be modulated

by increasing divalent cation concentration. The modulation of canavalin solubility is interesting from the viewpoint of protein chemistry. With increase in the divalent cation concentration, soluble canavalin decreased in the insolubilization phase, but increased in the solubilization phase. Therefore, canavalin insolubilization was altered to solubilization in a salt concentration-dependent manner. The phase transition is a unique property of canavalin. I assumed that the transition might be induced by the interaction between canavalin and non-proteinaceous substances or/and by some structural changes. However, the underlying molecular mechanism remains unclear, and further studies are needed.

The reversible change in solubility would be useful for the establishment of an inexpensive and simple method for the purification of canavalin. Studies on the primary structure of canavalin (Yamauchi *et al.*, 1988; Takei *et al.*, 1989) indicated that the 419 amino acid residues of the matured form of canavalin include 52 leucine residues. A previous study describes that the consumption of leucine in combination with exercise is effective in the prevention and improvement of sarcopenia (Katsanos *et al.*, 2006). It is also reported that, since whey proteins have relatively high leucine content (approximately 9.5 g of leucine per 100 g of proteins), the intake of whey proteins is effective to the prevention and improvement of sarcopenia (Yang *et al.*, 2012; Kuwata *et al.*, 1985). I estimated the leucine content in canavalin to be 14.3 g of leucine per 100 g of canavalin, which is approximately 1.5 times higher than that of whey proteins. The high leucine content of canavalin indicates that the intake of canavalin might be more effective than that of whey proteins. In addition, another study reports that the intake of animal proteins such as whey proteins increases carcinogenic risk (Kurahashi *et al.*, 2008). This risk would be reduced if leucine intake was via canavalin, a plant protein, rather than

via whey proteins. These findings indicate the desirability of using canavalin for the development of new processed foods.

In conclusion, I provided a schematic model of canavalin solubility changes induced by concentration changes of divalent cations in the crude extract. Canavalin solubility was affected by the addition of $MgCl_2$ or $CaCl_2$, but not of NaCl. Therefore, divalent cations play an important role in canavalin solubility. I also determined the C_m values for the insolubilization and solubilization phase of canavalin. Thus, these data provide important information regarding the physicochemical properties of canavalin in crude extracts of white sword bean that would be useful for the study of the physiological functions of canavalin and its application in food industry.

Chapter 3

Sword bean variants and different pretreatments influence protein extraction and protein properties

3.1. Introduction

Sword beans (*Canavalia gladiata*) have long been eaten in the Asian tropics and subtropics (Bressani *et al*., 1987; Siddhuraju and Becker, 2001). The average of yield of sword beans is comparable to that of soybeans, under optimal agricultural management conditions (Bressani *et al*., 1987). Sword beans are relatively resistant to pests and diseases (Smartt, 1976). From a nutritional perspective, the dried beans contain approximately 62% carbohydrate, 26% protein, and 3% fat (Vadivel and Janardhanan, 2005). The agricultural and nutritional characteristics show the potential for utilizing sword beans in processed foods. However, sword beans are little utilized for making processed foods. In Chapter 1 and 2, I have reported the physicochemical characteristics of sword bean proteins, which is important for making processed foods. However, more scientific knowledge is essential to begin using sword beans to make processed foods.

Sword bean is classified into two variant species, namely the white sword bean (WSB; Canavalia gladiata var. alba MAKINO) and red sword bean (RSB; Canavalia gladiata var. gladiata). Most previous reports have not described which beans were studied. When extracted substances from WSBs show different properties from the same substances extracted from RSBs, such a non-descriptive approach might impede a proper scientific understanding. In Chapter 1 and 2, dried WSBs were used for the protein extraction after water absorption by soaking. Dried WSBs absorbed sufficient water for extraction purposes by soaking in 10 volumes of distilled water for 18 h at 20°C. However,

Une *et al*. (2016) indicated that the water absorption of dried RSBs was very low after soaking. The different capacities for water absorption results in different pretreatments between WSBs and RSBs when preparing processed foods and cooking. The different pretreatments may have an influence on the extracted substances. A comparison between WSBs and RSBs is important for the scientific understanding of sword beans and the development of the processed foods from sword beans.

Previously, I reported that the solubility of canavalin, which is a major storage protein of sword bean with a molecular mass of 47.6 kDa (Yamauchi *et al.*, 1988; Takei *et al.*, 1989) and is classified as a 7S seed globulin or legume vicillin (Sumner *et al.*, 1983), is controlled by the $MgCl_2$ concentration in the crude extract from WSBs as shown in Chapter 2. The properties of extracted proteins are useful indicators for comparing the differences between WSBs and RSBs. In this study, water absorption was compared between WSBs and RSBs under the same conditions. In addition, drilled WSBs and RSBs were also compared in terms of water absorption. Sword bean proteins were extracted from WSBs and RSBs prepared by different pretreatments. To compare the $MgCl_2$ concentration-dependent control of canavalin solubility, $MgCl_2$ was added to the crude extracts at various concentrations. This study provides important insights into the scientific understanding of sword beans and the development of the processed foods from sword beans.

3.2. Materials and Methods

3.2.1. Materials

Dried WSBs and RSBs were purchased from Morika Kometen (Nara, Japan) and general chemical reagents were obtained from Wako Pure Chemical Industries Ltd. (Osaka,

Japan).

3.2.2. Measurement of bean size and weight

Bean sizes were measured with micrometer calipers (VC-15, AZONE, Osaka, Japan). The weights of beans were measured using an electronic balance (HR-120; A&D Company, Tokyo, Japan). Data are represented as the average ± standard deviation of 100 randomly selected beans.

3.2.3. Determination of soaking time

Soaking time was determined by a method as described in Chapter 1, with some modifications. Drilled beans were prepared by drilling four diagonal points using a 1-mm diameter drawing pin (Clear push pin, Moritoku, Osaka, Japan) to a depth of approximately 1 mm. Untreated and drilled beans were soaked in 10 volumes (v/w) of distilled water at 20°C for various durations. The size ratio was calculated by dividing the size of soaked beans by that of dried beans. The weight of the absorbed water was estimated by subtracting the initial weight of dried beans from that of soaked beans. The weight ratio was calculated by dividing the weight of the absorbed water by the initial weight of the dried beans. Data are represented as the average ± standard deviation of 5 beans.

3.2.4. Preparation of sword bean extracts

Sword bean extracts were prepared from soaked beans or bean flour as described below. Bean flour was prepared by grinding dried beans for 3 min with a grinder (Force Mill, Y-308B, OSAKA CHEMICAL Co., Ltd., Osaka, Japan). Soaked beans and bean flour were

ground in eight volumes (v/w) of distilled water (relative to the weight of dried beans and flour, respectively), with a hand blender (CSB-77JBSTRW, Cuisinart, CT, USA) on ice for 5 min. Each suspension was separated into an extract and waste by sieving through a cotton cloth.

3.2.5. Determination of protein concentration and amount

Protein concentrations were determined using the Bradford method with reagents from Bio-Rad Laboratories Inc. (CA, USA), using bovine serum albumin as a standard. The quantity of protein in each extract was calculated by multiplying the protein concentration by the extract volume. Data are represented as the average ± standard deviation of three independent experiments.

3.2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with 10% polyacrylamide gels at a constant current of 12.5 mA for 2.5 h, according to standard methods described by Laemmli (1970). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250. Samples were mixed with 0.33 volumes of an SDS buffer (0.25 M Tris-HCl at pH 7.0 containing 4% SDS, 5% 2-mercaptoethanol, and 40% glycerol). Prior to SDS-PAGE, the samples were incubated in boiling water for 5 min. A molecular weight standard was purchased from Life Technologies Japan Ltd. (Tokyo, Japan).

3.2.7. Analysis of supernatant proteins

Supernatant proteins were analyzed as described in Chapter 1 and 2. Sword bean extracts were incubated at 25°C for 5 min. Next, 0.11 volumes of $MgCl_2$ were added to each

incubated extract at various concentrations, mixed, and incubated at 25°C for 15 min and then on ice for 5 min. The mixtures were centrifuged at 9,100 × *g* for 20 min at 4°C. The supernatant proteins were analyzed by 10% SDS-PAGE.

3.2.8. Determination of the residual canavalin ratio

The residual canavalin ratio was determined by a method as described in Chapter 2. The intensity of canavalin bands on SDS-polyacrylamide gel was quantified by Image J software (National Institutes of Health, Bethesda, MD) (Abràmoff *et al.*, 2004). The residual canavalin ratio was expressed as the percentage of the canavalin band intensity for a sample incubated with $MgCl_2$ to that for a sample incubated with distilled water. Data are expressed as the average ± standard deviation of three independent experiments.

3.2.9. Statistical analyses

F-testing and t-testing were used to compare the mean sizes and weights between WSBs and RSBs in different the experiments. A one-way analysis of variance and Bartlett test were used to compared the means weights, protein concentrations, and quantities of extracted proteins among different experimental groups. A *post-hoc* analysis was performed with the Tukey－Kramer test if the analysis of variance revealed significance. Differences with $p < 0.05$ were considered statistically significant.

3.3. Results

3.3.1. Comparison of the sizes and weights of WSBs and RSBs

RSBs were larger than WSBs in appearance (Figure 3.1A). The sizes and weight of dried beans are summarized in Table 3.1. The long and minor axis sizes of RSBs were

significantly larger than those of WSBs ($p = 4.31 \times 10^{-6}$ for the long axis and $p = 2.20 \times 10^{-25}$ for the minor axis), but the thickness of RSBs was almost same as that of WSBs ($p = 0.0857$). RSBs were heavier than WSBs ($p = 1.06 \times 10^{-6}$). The sizes and weights of WSBs were almost the same as those of WSBs used in Chapter 1.

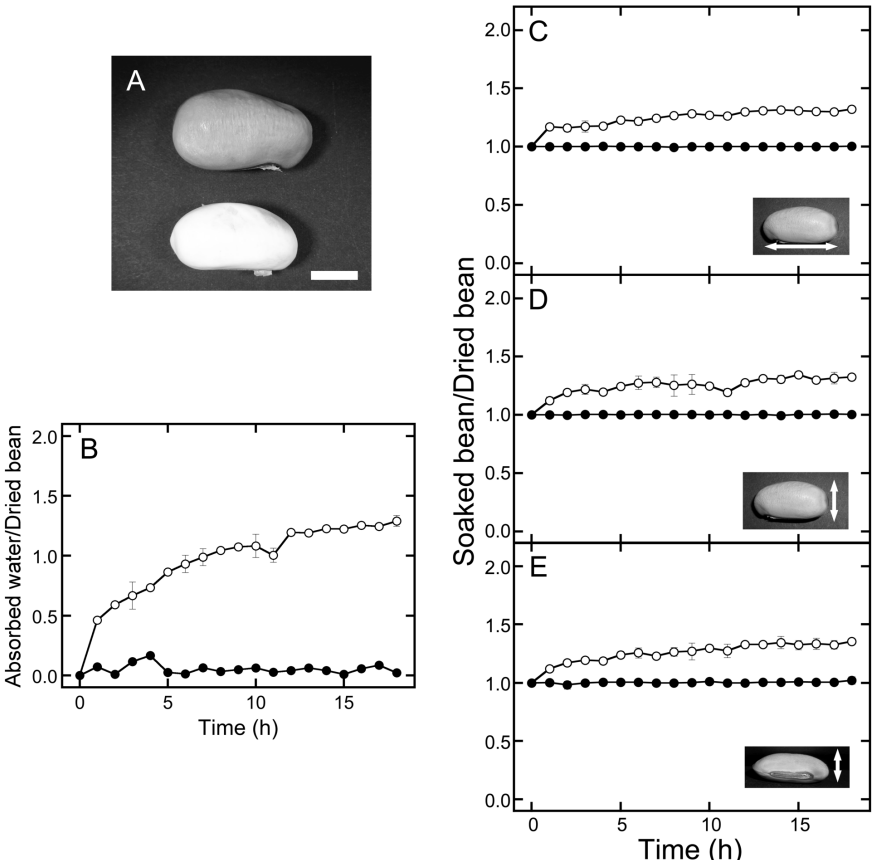

Figure 3.1 Size and weight transitions of sword beans due to water absorption during soaking.

(A) Appearance of an RSB (upper) compared with that of a WSB (lower). Scale bar, 1 cm. (B–E) WSBs (open circles) and RSBs (closed circles) were soaked in 10 volumes (v/w) of distilled water. (B) The weight of the absorbed water was estimated by subtracting the initial weight of the dried beans from that of the soaked beans. (C–E) The insets indicate the direction along which the sizes were measured. The double-headed arrows in panels C–E show the long axis (C), minor axis (D), and thickness (E). The weight ratio was calculated by dividing the weight of the absorbed water by the initial weight of the dried beans. The data are shown as the average ± standard deviation of 5 beans.

Table 3.1 Sizes and weights of dried sword beans.

	Long axis (cm)	Minor axis (cm)	Thickness (cm)	Weight (g)
WSB	2.72 ± 0.13	1.45 ± 0.08	1.16 ± 0.09	2.38 ± 0.36
RSB	2.99 ± 0.18*	1.69 ± 0.13*	1.14 ± 0.11	2.63 ± 0.37*

The data shown represent the average ± standard deviation of 100 randomly selected beans.
*, Differences with $p < 0.05$ between RSB and WSB determined by *t*-testing were considered statistically significant.

3.3.2. Changes of absorbed water weights and bean sizes during soaking

WSB proteins were extracted from sufficiently soaked beans, as described in Chapter 1. The absorbed water weight in the WSBs increased and nearly plateaued at 13 h (Figure 3.1B, opened circles). The increase was identical to that shown in Chapter 1. In contrast, the absorbed water weight in RSBs increased little over 18 h (Figure 3.1B, closed circles). Similarly, the WSB sizes increased, but the RSB sizes changed marginally (Figure 3.1C–E). The observed behaviors in RSB sizes and weights indicated that dried RSBs absorbed distilled water poorly without pretreatment. Une *et al.* (2016) also reported that the water absorption of untreated RSB was less than 5% after 24 h of soaking. Little water absorption in RSBs suggested that RSB proteins would be difficult to extract from RSBs using the method described in my previous study without some modifications.

3.3.3. Absorbed water-weight and bean-size changes of drilled sword beans

To promote the absorption of distilled water by RSBs, RSBs were drilled using a drawing pin at four positions (Figure 3.2A). The absorbed water weight in drilled RSBs dramatically increased and nearly plateaued at 12 h (Figure 3.2B, closed circles). In drilled WSBs, the absorbed water weight also drastically increased and nearly plateaued

at 12 h (Figure 3.2B, open circles). Drilled WSBs (closed circles) absorbed 1.3 times more water than untreated WSBs (dotted line) after an absorption time of 18 h (Figure 3.2B). The weight of absorbed water in drilled WSBs was almost the same as that of drilled RSBs. The increased water absorption may have resulted from water storage in the region between the seed coat and cotyledon.

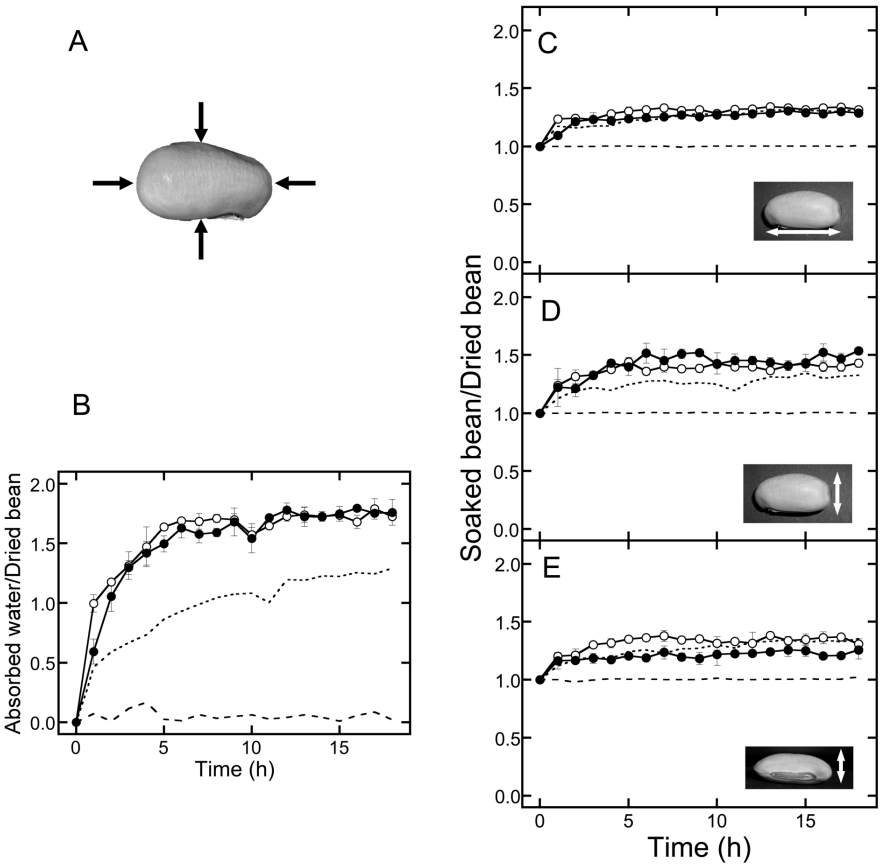

Figure 3.2 Effects of drilling on the bean size transition and water absorption.
(A) The four arrows show the positions of the holes that were drilled into the beans. (B–E) Drilled WSBs (open circles) and RSBs (closed circles) were soaked in 10 volumes (v/w) of distilled water. The data for the untreated WSBs (dotted line) and RSBs (dashed line) were taken from Figure 3.1. (C–E) The insets indicate the direction along which the sizes were measured. The double-headed arrows in panels C–E show the long axis (C), minor axis (D), and thickness (E). The ratio was calculated as described in Figure 3.1. The data are shown as the average ± standard deviation of 5 beans.

The sizes of RSBs increased by soaking after drilling (Figure 3.2C–E, closed circles). The long axis length in drilled RSBs increased more slowly than that of drilled WSBs, but reached a similar maximum level, compared with that of drilled and untreated WSBs (Figure 3.2C). The minor-axis dimension in RSBs also increased to a similar maximum level, compared with that of drilled WSBs, and the maximum minor axis sizes in RSBs and WSBs were larger than that of untreated WSBs (Figure 3.2D). The thickness of RSBs also increased (Figure 3.2E). These results indicate that drilling promoted efficient water absorption and that water absorption was inhibited by the seed coat of RSBs.

3.3.4. Preparation of extracts following different pretreatments

To extract proteins from beans in water, we prepared untreated, drilled, and milled beans. Untreated WSBs, drilled WSBs, and drilled RSBs were ground in eight volumes (v/w) of distilled water following soaking for 18 h in distilled water. Milled beans were also ground in eight volumes (v/w) of distilled water in the same manner without soaking in advance. Suspensions were separated into extracts and wastes. These yields were quantitatively estimated, as summarized in Table 3.2. The yields of suspensions from drilled WSBs and RSBs were a little larger than that from untreated WSBs. The increased yield could have resulted from the increases water absorption (Figure 3.2B). In contrast, the yields of suspensions from milled WSBs and RSBs were significantly smaller than that from untreated WSBs (Table 3.2). The decrease yield appeared to be induced by the water absorption of the dried sword bean powder during the preparation. The yield of extract from drilled beans was significantly larger than that from untreated WSBs and milled beans (Table 3.2), which may have resulted from the abundant water absorption

(Figure 3.2B). The yield of waste from milled beans was smaller than that from untreated WSBs and drilled beans (Table 3.2). The finer powder produced from milled beans likely caused the decrease, considering that waste was separated by sieving through a cotton cloth. The collection rate was over 80% with either type of bean (Table 3.2). The lower yield was related to water absorption to the cotton cloth.

Table 3.2 Comparison of extract weights between beans prepared with different pretreatments.

	Suspension (g/g bean)	Extract (g/g bean)	Waste (g/g bean)	Collection rate (%)
WSBs				
Untreated	9.0 ± 0.3^{a}	$5.6 \pm 0.1^{b,c}$	1.8 ± 0.3^{a}	82.2
Drilled	9.6 ± 0.6^{a}	7.2 ± 0.4^{a}	$1.5 \pm 0.2^{a,b}$	90.6
Milled	7.5 ± 1.0^{b}	5.1 ± 0.8^{c}	1.1 ± 0.1^{b}	82.7
RSBs				
Untreated	–	–	–	–
Drilled	9.5 ± 0.1^{a}	$6.6 \pm 0.3^{a,b}$	$1.6 \pm 0.2^{a,b}$	86.3
Milled	7.5 ± 0.3^{b}	4.9 ± 0.1^{c}	1.2 ± 0.1^{b}	81.3

Values are expressed as means ± SD for three different experiments. Means within the same column bearing different superscripted roman letters are significantly different, with $p < 0.05$ determined by analysis of variance using the Tukey–Kramer test. The collection rate was estimated by dividing the total extract and waste weight by the weight of the suspension.

Table 3.3 Protein concentrations and quantities of sword bean extracts.

	Protein concentration (mg/mL)	Protein quantity[1] (mg protein/g dried bean)
WSBs		
Untreated	16.6 ± 1.2[a]	93.0 ± 6.7[a,b]
Drilled	13.1 ± 1.1[b,c]	94.0 ± 7.9[a]
Milled	14.5 ± 0.6[a,b]	74.1 ± 3.1[c]
RSBs		
Untreated	–	–
Drilled	11.7 ± 0.9[c]	77.3 ± 5.9[b,c]
Milled	13.2 ± 1.2[b,c]	52.7 ± 4.8[d]

[1]Protein quantities in the extract were determined by multiplying the protein concentration by the extract volume. Values are expressed as means ± SD for three different experiments. Means within the same column bearing different superscripted roman letters are significantly different, with $p < 0.05$ determined by analysis of variance using the Tukey–Kramer test.

3.3.5. Extracted proteins from beans following different pretreatments

Proteins were extracted from WSBs and RSBs. The protein concentrations are summarized in Table 3.3. The protein concentration in untreated WSBs was higher than that in other WSBs and RSBs. In addition, the protein concentration in drilled beans were lower than that in milled beans. Protein quantities were also calculated (Table 3.3) by multiplying the protein concentration by the volume of the extract shown in Table 3.2. The quantity of extracted proteins in milled RSBs was lowest and approximately half of that in untreated WSBs. When comparing WSBs and RSBs prepared using the same pretreatment, the quantity of proteins extracted from RSBs was lower than that of WSBs. However, the protein quantity in drilled WSBs was almost the same as that in untreated WSBs. These results showed that proteins were extracted from untreated WSBs and drilled WSBs with similar efficiency and that the efficiency in milled beans was lower

than that of untreated and drilled beans. In addition, although the quantity of extracted protein between untreated WSBs and milled WSBs was almost the same ($p > 0.5$), the protein concentration obtained from drilled WSBs was clearly lower than that from milled WSBs ($p = 0.012$). The lower protein concentration obtained from drilled WSBs supports the possibility that water was stored in the region between the seed coat and cotyledon when soaking the drilled beans. Furthermore, the composition of extracted proteins was analyzed by SDS-PAGE (Figure 3.3). The protein patterns were nearly identical between the extracts from beans prepared by different pretreatments. The protein-concentration, protein-quantity, and SDS-PAGE results indicated that using untreated WSB is better suited for protein extraction.

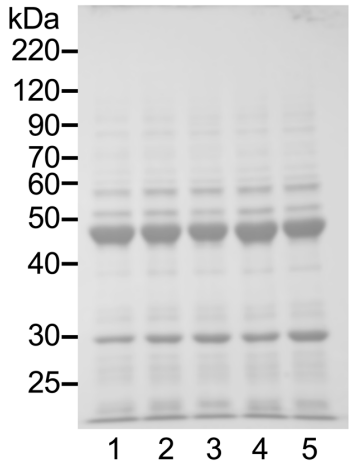

Figure 3.3 SDS-PAGE analysis of RSB and WSB proteins extracted by different procedures.

Sword bean proteins were separated by SDS-PAGE (10% polyacrylamide). Ten micrograms of sword bean proteins from each extract were electrophoresed and stained with Coomassie Brilliant Blue R-250. Extracts were prepared from soaked WSBs (lane 1), drilled and soaked WSBs (lane 2) and RSBs (lane 3), and milled WSBs (lane 4) and RSBs (lane 5).

3.3.6. Influence of different pretreatments on canavalin solubility

Canavalin solubility is controlled by the divalent cation concentration in a crude extract as shown in Chapter 2. To investigate the influence of different pretreatments, $MgCl_2$ was added to each extract in the range of 0–50 mM. The mixtures were divided into insoluble and soluble phases. The soluble phase was analyzed by SDS-PAGE (Figure 3.4). The canavalin solubility was controlled by the $MgCl_2$ concentration in a concentration-dependent manner, where canavalin tended to be insoluble at relatively low $MgCl_2$ concentrations and solubilize at relatively high concentrations, as reported in Chapter 2. However, with different pretreatments, the behaviors appeared something like different control by the $MgCl_2$ concentration.

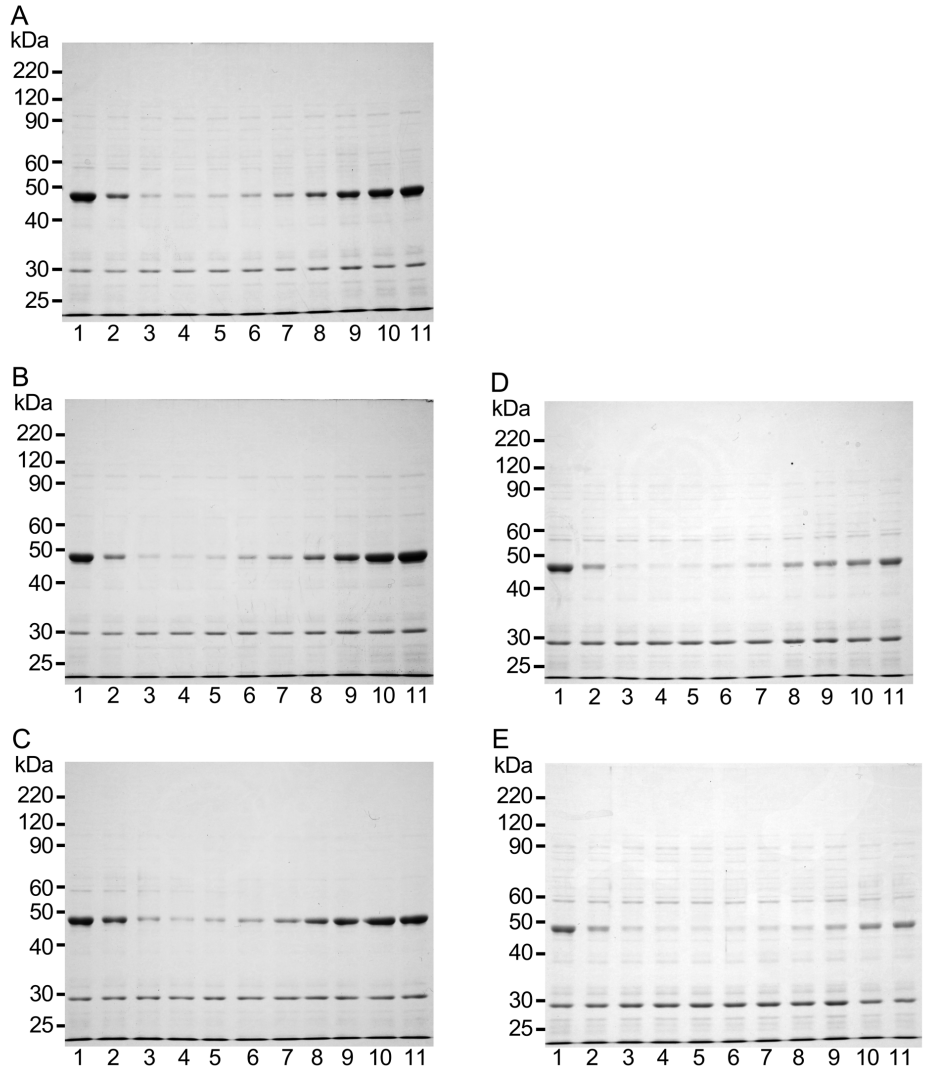

Figure 3.4 Canavalin solubility changes observed by adding $MgCl_2$ to different extracts. Different extracts were prepared from soaked WSBs (A), drilled and soaked WSBs (B), milled WSBs (C), drilled and soaked RSBs (D), and milled RSBs (E). $MgCl_2$ was added to extracts prepared by different procedures at a final concentration of 5 mM (lane 2), 10 mM (lane 3), 15 mM (lane 4), 20 mM (lane 5), 25 mM (lane 6), 30 mM (lane 7), 35 mM (lane 8), 40 mM (lane 9), 45 mM (lane 10), or 50 mM (lane 11). Distilled water was used instead of $MgCl_2$ as a control (lane 1). Five micrograms of sword bean proteins from each extract were electrophoresed on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

To compare the effects of different pretreatments in detail, residual canavalin was quantified based on the band intensities found by SDS-PAGE analysis (Figure 3.5). Compared with canavalin in extracts from untreated WSB, canavalin in extracts from milled WSBs showed greater insolubility with a higher $MgCl_2$ concentration and greater solubility at lower $MgCl_2$ concentrations (Figure 3.5A, open squares). In contrast, canavalin from drilled WBSs was showed greater insolubility at lower $MgCl_2$ concentrations and greater solubility at higher $MgCl_2$ concentrations, compared with that of untreated WSBs (Figure 3.5A, open triangles). In addition, canavalin from drilled and milled RSBs showed similar insolubility compared to canavalin from untreated WSBs, but was solubilized by a higher $MgCl_2$ concentration than canavalin from untreated WSBs (Figure 3.5B). The different controls indicate that the different bean pretreatments influenced canavalin solubility based on the $MgCl_2$ concentration. Interestingly, the standard deviations for drilled and milled beans were larger than that for untreated WSBs. The difference in the standard deviation implies that extracts from untreated WSBs were of uniformly higher quality than those from drilled and milled beans.

To compare the controls between WSB and RSB, the canavalin solubilities were replotted for the drilled and milled beans (Figure 3.6). With drilled beans, canavalin from WSBs showed comparable insolubility to that from RSBs, but was solubilized by a lower $MgCl_2$ concentration than that from RSBs (Figure 3.6A). In contrast, in milled beans, canavalin from WSBs was insolubilized by a slightly higher $MgCl_2$ concentration than that from RSBs, but was solubilized by a much lower $MgCl_2$ concentration than that from RSBs (Figure 3.6B). These results indicate that the solubility of canavalin from WSBs and RSBs was controlled by the $MgCl_2$ concentration in different manners.

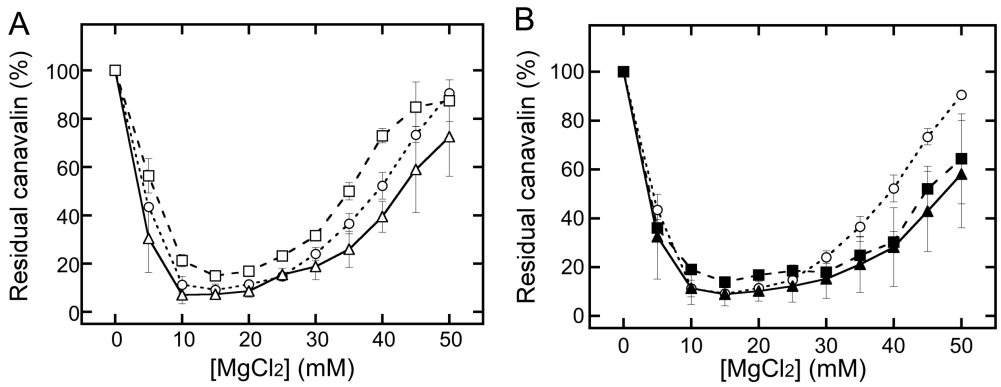

Figure 3.5 Comparison of canavalin solubility changes between different extracts.

The canavalin solubility in the extracts shown in Figure 3.4 was analyzed by SDS-PAGE after adding $MgCl_2$ to WSB (A) and RSB (B) extracts. The proportion of residual canavalin in the supernatant was estimated from band intensity of Figure 3.4 using ImageJ software. Open and closed symbols indicate WSBs and RSBs, respectively. The circles and dotted line indicate data for control WSBs that were only treated by soaking. The triangles and solid line indicate drilled and soaked beans. The squares and dashed line represents milled beans.

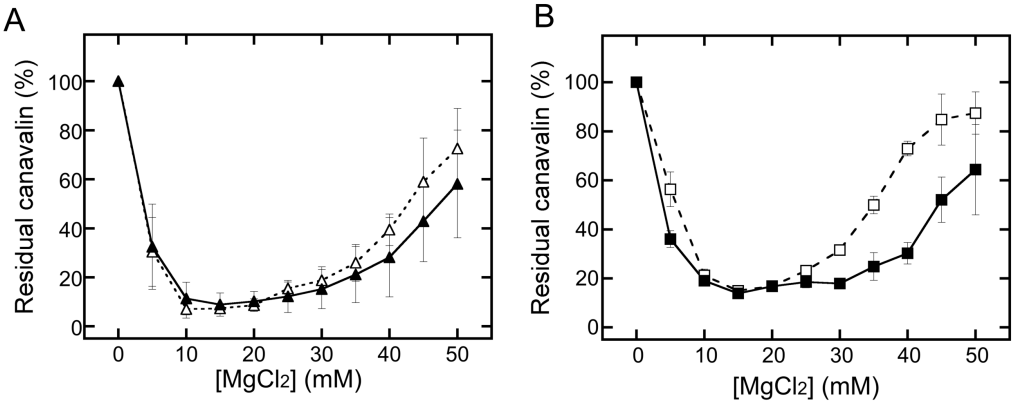

Figure 3.6 Comparison of canavalin solubility changes between WSBs and RSBs.

Samples were prepared from drilled and soaked beans (A) and milled beans (B). The data shown were replotted from the data in Figure 3.5 to compare WSBs (open triangles and squares) and RSBs (closed triangles and squares).

3.4. Discussion

Untreated RSBs absorbed little distilled water during a long soaking period. Large quantities of sword bean proteins were extracted from beans prepared after different pretreatments. Proteins were extracted with high efficiency from untreated and drilled beans. The protein-concentration, protein-quantity, and SDS-PAGE data indicated that the use of untreated WSBs was favorable for protein extraction. The canavalin solubility was controlled by the $MgCl_2$ concentration in a similar manner as shown in Chapter 2. However, the behavior was distinctly different between extracts prepared with different pretreatments. In addition, the solubility of canavalin from RSB and WSBs was controlled in different manners by the $MgCl_2$ concentration. These results indicate that method used to prepare beans influences the protein properties in a crude extract. The bean variety and pretreatment are also important factors for using beans for food chemical experiments and biochemical experiments.

Chapter 4

Structural transitions of sword bean canavalin in response to different salt concentrations

4.1. Introduction

The sword bean (*Canavalia gladiata*) is an edible leguminous plant that originated either from southern Asia or Africa (Purseglove, 1968). Sword bean seeds are highly nutritious and contain ~26% protein (Vadivel and Janardhanan, 2005). Considering their agronomical and nutritional features, sword beans are expected to be ideal for use in processed foods as a source of protein. The major protein is canavalin, which belongs to the 7S seed globulin, or vicilin, class with a molecular weight of 47.6 kDa (Sumner *et al*., 1983).

In Chapter 1, I established a method to extract the proteins from dried sword bean seeds in distilled water. Further, its solubility can be reversibly altered via the addition of Mg^{2+} and Ca^{2+} at different concentrations as shown in Chapter 2. Moreover, canavalin that is extracted in distilled water is soluble and becomes precipitated following the addition of low concentrations of divalent cations; however, this effect is lost in the presence of higher concentrations of divalent cations. Precipitated canavalin ($MgCl_2$-precipitated canavalin) is reversibly resolubilized in the presence of high concentrations of divalent cations, but is not solubilized in distilled water. In addition, NaCl can maintain canavalin in the soluble form in sword bean extracts, regardless of the NaCl concentration (0–400 mM) as shown in Chapter 2. The variable solubility of canavalin is an interesting physicochemical property. However, it is unclear whether the addition of NaCl can solubilize $MgCl_2$-precipitated canavalin and whether the structures of these soluble

proteins differ.

In this study, NaCl was added to $MgCl_2$-precipitated canavalin to investigate the ability of NaCl to solubilize $MgCl_2$-precipitated canavalin. In addition, the quaternary structures of canavalin were investigated under different conditions using gel filtration. The findings from this study have the potential to serve as an important reference for the analysis of sword bean protein characteristics and 7S globulin characteristics.

4.2. Materials and Methods

4.2.1. Materials

White sword beans were purchased from Morika Kometen (Nara, Japan), and general chemical reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). The molecular weight standard kits (LMW and HMW calibration kits) were purchased from GE Healthcare UK Ltd (Little Chalfont, England).

4.2.2. Preparation of the sword bean extract

The sword bean extract was prepared according to previously described methods in Chapter 1. Dried sword beans were soaked in 10 volumes (v/w) of distilled water at 20 °C for 18 h. The soaked beans were then ground on ice for 5 min in 8 volumes (v/w) of distilled water using a hand blender (CSB-77JBSTRW, Cuisinart, Stamford, CT, USA). The suspension was sieved through a cotton cloth. The extract was centrifuged at 9,100 × *g* for 10 min at 4 °C, and the supernatant was used in the analyses.

4.2.3. Analysis of sword bean proteins

The solubility of sword bean proteins was analyzed using previously described methods

as shown in Chapter 1 and 2 with specific modifications. Briefly, 150 mM $MgCl_2$ or 2 M NaCl (0.1 mL) was added to 9 volumes of sample extract (0.9 mL) and incubated at 25 °C for 15 min and on iced for 5 min. Samples were then separated into supernatant and precipitate via centrifugation at 9,100 × *g* at 4 °C for 20 min. The precipitate (prepared by adding $MgCl_2$ to the sword bean extract) was suspended in 60 mM $MgCl_2$ or 200 mM NaCl. The mixtures were again separated into the supernatant and precipitate by centrifugation at 9,100 × *g* at 4 °C for 20 min. The precipitates were dissolved in a volume of 8 M urea in a volume equal to that of the salt-added extract (1.0 mL). Sword bean proteins were separated by gel filtration chromatography and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.4. SDS-PAGE

Samples (90 μL) were mixed with 0.33 volumes (30 μL) of SDS sample buffer (0.25 M tris-HCl [pH 7.0], 4% SDS, 5% 2-mercaptoethanol, and 40% glycerol) and incubated at 100 °C for 5 min. SDS-PAGE was conducted with 10% polyacrylamide gels at a constant current of 12.5 mA for 2.5 h, according to the standard method described by Laemmli (1970). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250. The molecular weight standard was purchased from Life Technologies (Tokyo, Japan).

4.2.5. Determination of the residual canavalin ratio

Residual canavalin in the supernatant was quantified using previously described methods in Chapter 2. The intensity of canavalin bands on the SDS-polyacrylamide gel was quantified by ImageJ (National Institutes of Health, Bethesda, MD) (Abràmoff *et al.*, 2004). The residual canavalin ratio was expressed as the percentage of band intensity of

the NaCl-added samples to that of the samples with distilled water. Data were expressed as means ± standard deviations (SDs) of three independent replicates.

4.2.6. Gel filtration chromatography

Sword bean proteins were separated using a gel filtration column (HiPrep 16/60 Sephacryl S-200 High Resolution, GE Healthcare UK Ltd, England) with a bed volume of 120 mL in distilled water containing 200 mM NaCl or 60 mM $MgCl_2$ at a flow rate of 0.5 mL/min. Samples were injected into the column in volumes of 2.0 mL. Eluted samples were collected in volumes of 1.5 mL. The molecular weight standard was prepared by mixing the LMW calibration kit with aldose from the HMW calibration kit. The standard contained aprotinin, ribonuclease A, carbonic anhydrase, ovalbumin, conalbumin, and aldolase with average molecular masses of 6,500, 13,700, 29,000, 44,000, 75,000, and 158,000 Da, respectively. The collected samples were assayed using Bradford dye reagent (Bio-Rad Laboratories Inc., CA, USA) according to the commercial protocol. Absorbance was measured at 595 nm.

4.3. Results

4.3.1. Effects of NaCl on canavalin solubility

To investigate the effect of NaCl on $MgCl_2$-precipitated canavalin, the $MgCl_2$-precipitated canavalin was suspended in a high concentration of NaCl (Figure 4.1). Canavalin was observed in the supernatant (lane 2) but not in the precipitate (lane 3). These results indicated that canavalin was soluble when the concentration of NaCl was high. In contrast, when $MgCl_2$ was added to the sword bean extract at a final concentration of 15 mM, most canavalin was observed in the precipitate (lane 5) but not in the

supernatant (lane 4) when the concentration of $MgCl_2$ was low. Furthermore, the $MgCl_2$-precipitated canavalin (lane 5) was suspended in 200 mM NaCl. In the suspension, most canavalin was observed in the supernatant (lane 6) rather than the precipitate (lane 7). The results indicated that the $MgCl_2$-precipitated canavalin was solubilized via the addition of 200 mM NaCl.

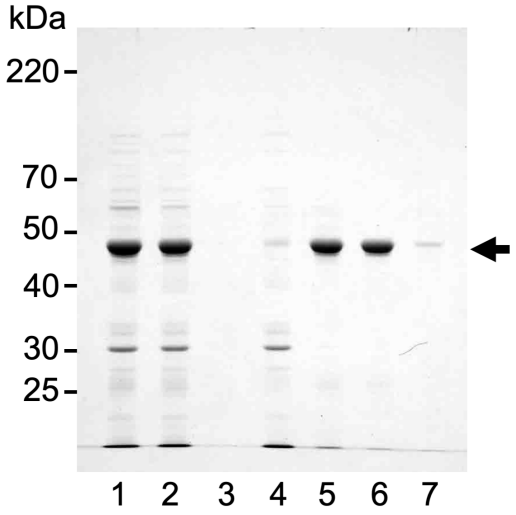

Figure 4.1 Effect of NaCl on canavalin solubility.

NaCl was added to the sword bean extract (lane 1) at a final concentration of 200 mM. The NaCl mixture was separated into the supernatant (lane 2) and precipitate (lane 3) by centrifugation. $MgCl_2$ was also added to the sword bean extract at a final concentration of 15 mM. The $MgCl_2$ mixture was separated into the supernatant (lane 4) and precipitate (lane 5) by centrifugation. The precipitate from the $MgCl_2$ mixture was suspended in 200 mM NaCl. The suspension was again separated into the supernatant (lane 6) and precipitate (lane 7) by centrifugation.

4.3.2. NaCl concentration-dependent changes in canavalin solubility

The NaCl concentration-dependency of canavalin solubilization was analyzed by suspending $MgCl_2$-precipitated canavalin (Figure 4.2A, lane 1) in various concentrations of NaCl (range: 0–200 mM, Figure 4.2). The mixtures were then separated into supernatants and precipitates (Figure 4.2A, lanes 2-12). The intensity of the canavalin bands indicated the amount of canavalin present (Figure 4.2B). Soluble canavalin gradually increased with increasing NaCl concentrations until it reached a maximum (102.5 ± 7.9%) in the presence of 180 mM NaCl. These results indicated that the solubility of $MgCl_2$-precipitated canavalin was NaCl concentration-dependent and that the precipitated canavalin was almost entirely solubilized at high concentrations of NaCl.

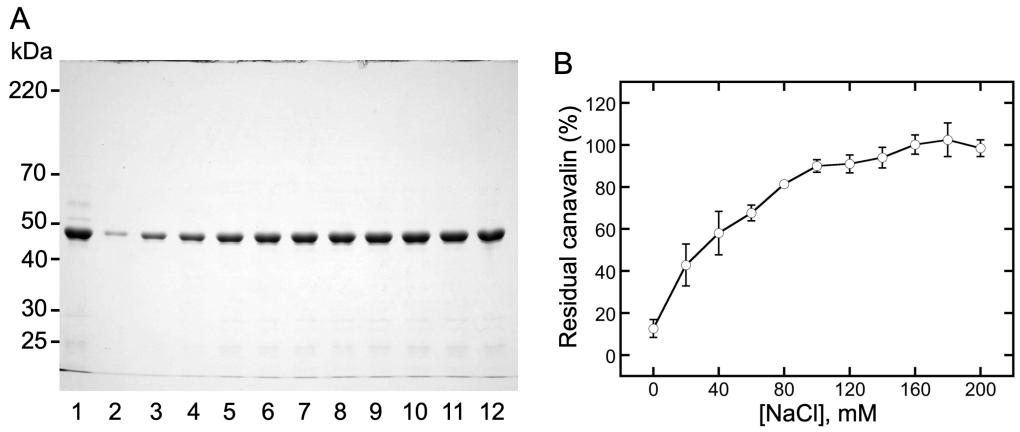

Figure 4.2 Effect of NaCl concentration on canavalin solubility.

(A) Canavalin in the sword bean extract was precipitated via the addition of $MgCl_2$ at a final concentration of 15 mM and then suspended in 8 M urea (lane 1), distilled water (lane 2), or NaCl at concentrations of 20 mM (lane 3), 40 mM (lane 4), 60 mM (lane 5), 80 mM (lane 6), 100 mM (lane 7), 120 mM (lane 8), 140 mM (lane 9), 160 mM (lane 10), 180 mM (lane 11), and 200 mM (lane 12). Distilled water was used as the control (0 mM). Suspensions were separated into the supernatant and precipitate, and the supernatant was subjected to SDS-PAGE (10% polyacrylamide). (B) The proportion of residual canavalin in the supernatant was estimated from the band intensity using ImageJ. Data are expressed as the means ± standard deviations of three independent replicates.

4.3.3. Structural differences between soluble canavalin in sword bean extracts and in high concentrations of NaCl

As described in Chapter 2, soluble canavalin in sword bean extracts can be maintained in a soluble form via the addition of low concentrations of NaCl. However, as shown in Figure 4.2, almost half of the $MgCl_2$-precipitated canavalin was insolubilized in the presence of NaCl at concentrations < 60 mM. This inconsistency implies that although canavalin in sword bean extracts and in solutions with high concentrations of NaCl have similar soluble forms, their structures differ.

To detect these structural differences, soluble canavalin in sword bean extract (Figure 4.3) and in a high-concentration NaCl solution (Figure 4.4) was analyzed by gel filtration chromatography. Proteins in the crude extract were clearly eluted from fraction numbers 35 to 80 (Figure 4.3A). The abundance of eluted proteins was observed to increase in two primary stages. In the first stage, the abundance (equivalent to absorbance) was seen to steadily increase beginning at fraction 35 through to fraction 43, at which point it plateaued until fraction 49. In the second stage, protein abundance consistently increased from fraction 49 to 52 and plateaued from 52 to 55 after which the protein abundance was seen to steadily decrease until fraction 80. The peak abundance was determined to occur in fraction 51. SDS-PAGE analysis indicated that the molecular weight of the major eluted protein was approximately 47 kDa (Figure 4.3B). The theoretical molecular weight of canavalin is ~47.6 kDa (Sumner *et al.*, 1983). These results indicate that a large proportion of the eluted proteins comprised canavalin. The molecular weight standard was also eluted under the same conditions. According to an estimate based on the elution pattern of the molecular weight standard, proteins with this molecular weight are theoretically eluted at fraction number 39. As shown in the elution

pattern and SDS-PAGE analysis, the elution position of canavalin was broad, although it was also eluted at fraction number 39. These results indicate that the canavalin in sword bean extract was eluted in a position that corresponds to a lower molecular weight monomeric form. Hence, from the elution position, it was deduced that canavalin existed as a monomer and in an unstable form within the crude extract.

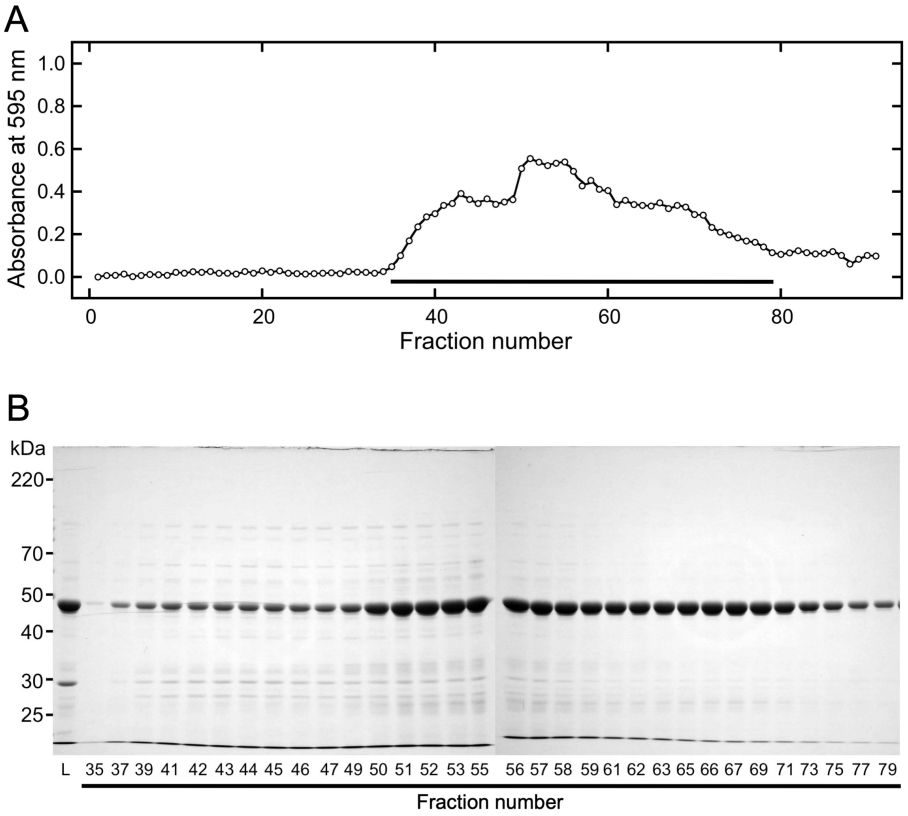

Figure 4.3 Gel filtration chromatography of proteins extracted from sword beans. Extracted proteins in distilled water (2 mL) were loaded onto a gel filtration column that was equilibrated with distilled water at a flow rate of 0.5 mL/min. Eluted samples were collected in volumes of 1.5 mL. (A) After gel filtration chromatography, each eluted sample was reacted with Bradford dye reagent and then measured at 595 nm. Black bars indicate fractions analyzed by SDS-PAGE. (B) The loaded (L) and eluted samples were subjected to SDS-PAGE (10% polyacrylamide). Numbers represent fraction numbers in panel A. Eluted samples were distinguished between fraction numbers 35–55 and 56–79 on different gel plates.

In the high-concentration solution, proteins were eluted primarily from fraction 25 to 39 (Figure 4.4A). The peak abundance was determined to occur in fraction 31. Moreover, SDS-PAGE analysis indicated that the majority of eluted proteins were canavalin (Figure 4.4B). In previous studies, crystal structures of canavalin indicated that canavalin occurred in its homo-trimer form (McPherson, 1980; Ko *et al.*, 2001). The hypothetical molecular weight of trimeric canavalin is 142.8 kDa and proteins of this size are theoretically eluted in fraction number 32. Although fraction number 31 was estimated to be have a molecular weight of 178.1 kDa, the elution position indicated that the $MgCl_2$-precipitated canavalin suspended in 200 mM NaCl was in its trimer form. In addition, the results presented in Figures 4.3 and 4.4 indicate that the canavalin in the sword bean extract was structurally different from that in the 200 mM NaCl solution, which was prepared by solubilizing $MgCl_2$-precipitated canavalin.

As shown in Chapter 2, soluble canavalin in the sword bean extract remained in a soluble form via the addition of NaCl. To investigate the effect of directly changing the NaCl concentration on the quaternary structure of canavalin, a sample was prepared by adding NaCl to the sword bean extract at a final concentration of 200 mM. Proteins in the sample were then separated by gel filtration chromatography (Figure 4.5). Eluted samples were analyzed in the same way as previously described (Figure 4.5A). The abundances. of eluted proteins were dramatically increased in fraction number 25, with peak abundance measured in fraction 30. The pattern of protein abundance was similar to that observed for soluble canavalin prepared from $MgCl_2$-precipitated canavalin. However, the decrease in eluted proteins was comparatively more gradual than that observed with the soluble form prepared from $MgCl_2$-precipitated canavalin. SDS-PAGE analysis indicated that the main eluted protein was canavalin and that similar protein

concentrations were eluted in fractions 30 to 32 (Figure 4.5B). These results indicated that canavalin underwent alterations in its structure to form a trimer following the addition of high concentrations of NaCl.

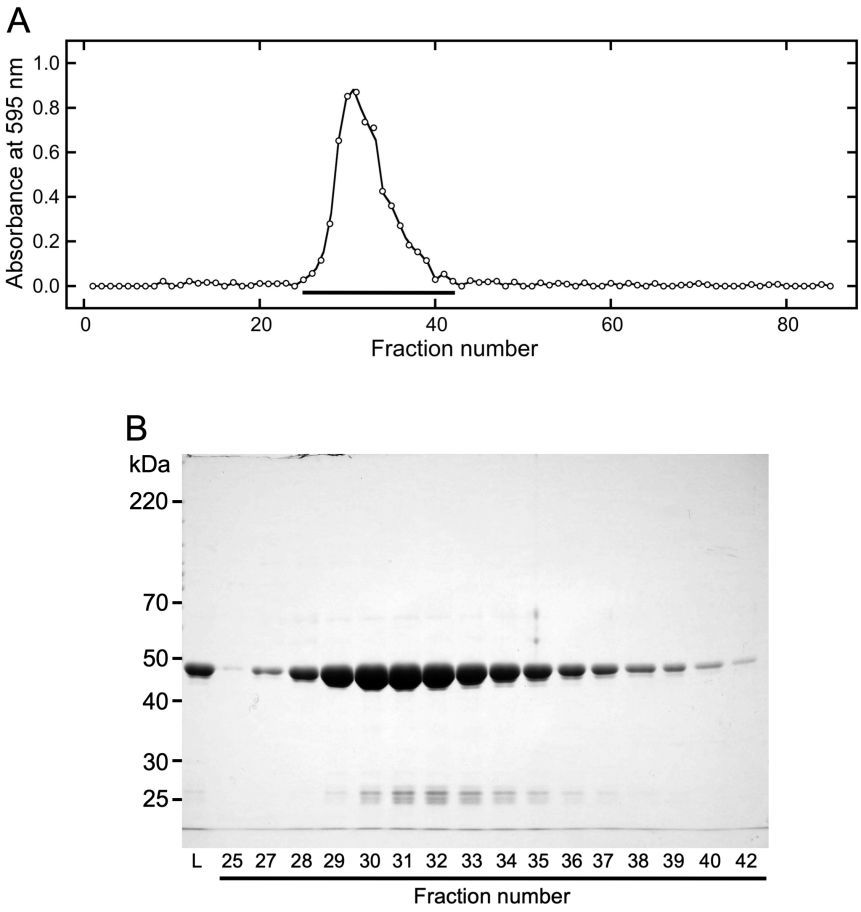

Figure 4.4 Gel filtration chromatography of proteins in the sword bean extract and NaCl mixture.

NaCl was added to the sword bean extract at a final concentration of 200 mM. The NaCl mixture was separated into the supernatant and precipitate by centrifugation. Supernatant proteins (2 mL) were loaded onto a gel filtration column that was equilibrated with 200 mM NaCl at a flow rate of 0.5 mL/min. Eluted samples were collected in volumes of 1.5 mL. (A) Each eluted sample was reacted with Bradford dye reagent and then measured at 595 nm. Black bars indicate fractions analyzed by SDS-PAGE. (B) The loaded (L) and eluted samples were subjected to SDS-PAGE (10% polyacrylamide). Numbers represent fraction numbers in panel A.

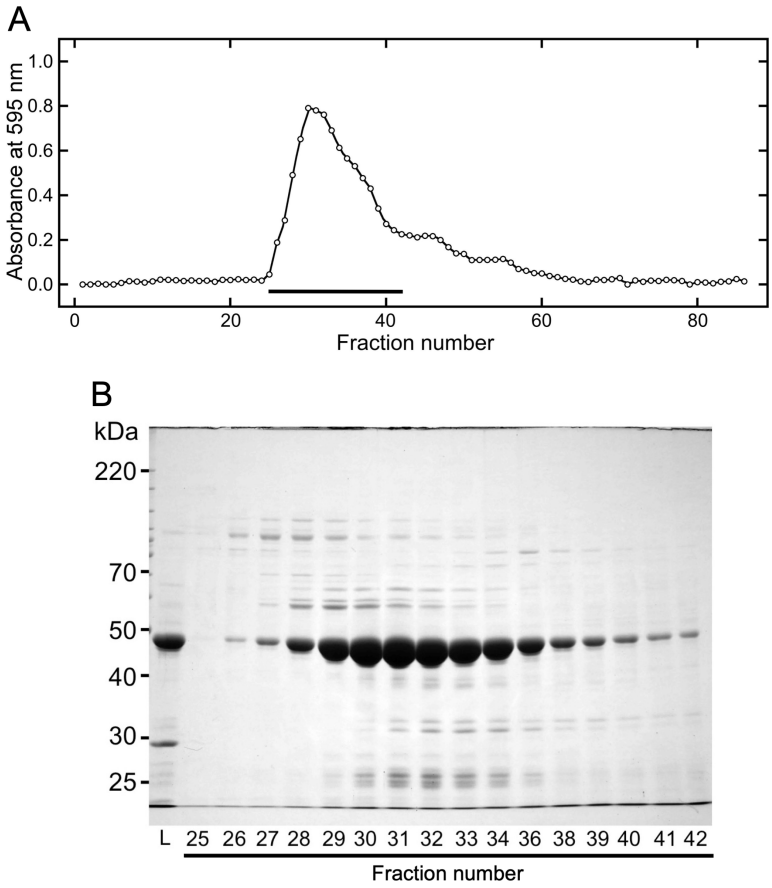

Figure 4.5 Gel filtration chromatography of proteins in the suspension with NaCl.
Canavalin in the sword bean extract was precipitated via the addition of $MgCl_2$ at a final concentration of 15 mM. Precipitated canavalin was suspended in 200 mM NaCl, and the suspension was separated into the supernatant and precipitate by centrifugation. Supernatant proteins (2 mL) were loaded onto a gel filtration column that was equilibrated with 200 mM NaCl at a flow rate of 0.5 mL/min. Eluted samples were collected in volumes of 1.5 mL. (A) The eluted samples were reacted with Bradford dye reagent and then measured at 595 nm. Black bars indicate fractions analyzed by SDS-PAGE. (B) The loaded (L) and eluted samples were subjected to SDS-PAGE (10% polyacrylamide). Numbers represent fraction numbers in panel A.

4.3.4. Elution pattern of canavalin in the presence of high-concentration $MgCl_2$

To investigate the effects of $MgCl_2$ on the quaternary structure of canavalin, it was added to the sword bean extract at a final concentration of 60 mM, at which canavalin was in a soluble form. Supernatant proteins were separated by gel filtration chromatography (Figure 4.6). Eluted proteins increased in abundance beginning at fraction 24 with the peak abundance measured in fraction number 33. The elution pattern strongly suggested that the soluble canavalin that was prepared from sword bean extract with the addition of $MgCl_2$ was also in a trimer form. SDS-PAGE analysis indicated that the primary eluted protein was canavalin (Figure 4.6B). However, the abundance of eluted canavalin was much lower than that eluted in distilled water and 200 mM NaCl. Furthermore, the resulting molecular weights of eluted proteins (> 220 kDa) suggested that proteins formed aggregates in the samples from fraction 30 to 39. These results indicate that canavalin might more readily aggregate in the presence of $MgCl_2$ compared to that in NaCl (Figures 4.4 and 4.5).

As described in Chapter 2, $MgCl_2$-precipitated canavalin can be solubilized in 60 mM $MgCl_2$. To investigate the effect of $MgCl_2$ on the quaternary structure of canavalin, resolubilized canavalin was prepared by solubilizing the $MgCl_2$-precipitated canavalin in 60 mM $MgCl_2$. Then, supernatant proteins were separated by gel filtration chromatography (Figure 4.7) and eluted samples were analyzed as described previously (Figure 4.7A). The abundance of eluted proteins increased beginning in fraction 26 and the peak abundance was observed in fraction 32. These results indicated that the soluble canavalin prepared by adding $MgCl_2$ to the $MgCl_2$-precipitated canavalin also existed as a trimer. Eluted samples were subjected to SDS-PAGE (Figure 4.7B) and the main eluted protein was canavalin. However, as also seen in Figure 4.6, the amount of eluted canavalin

was much lower than that after elution in distilled water and 200 mM NaCl; moreover, proteins in the sample formed aggregates in fractions 30 to 39. These results confirm that canavalin more readily forms aggregates in the presence of $MgCl_2$ compared to that in solutions with NaCl (Figures 4.4 and 4.5).

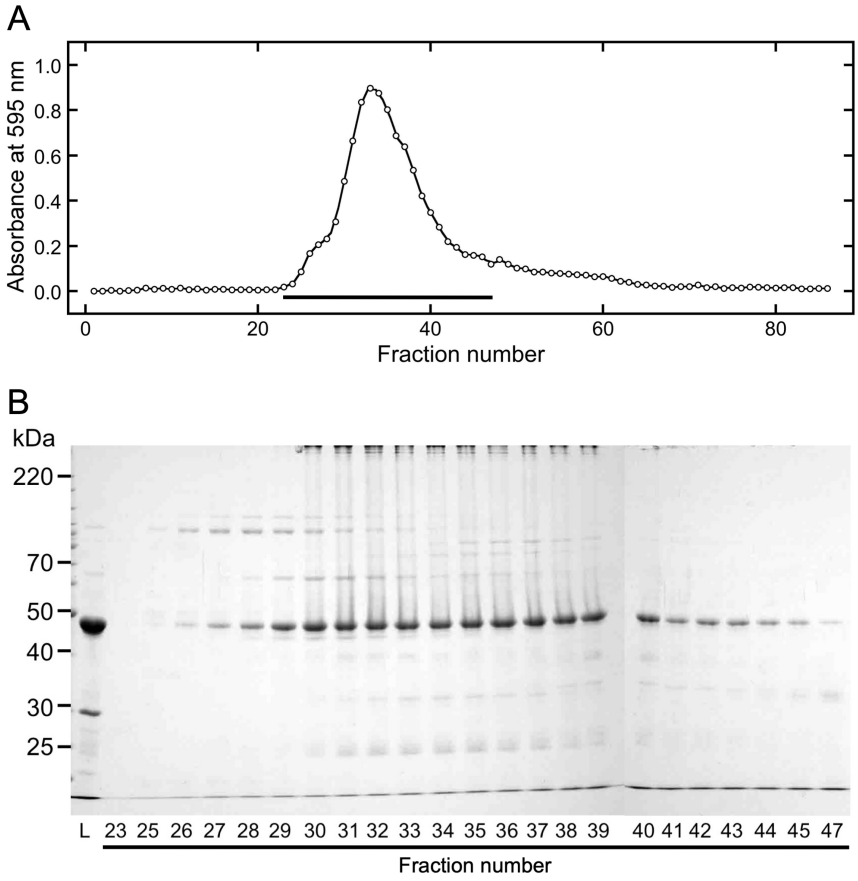

Figure 4.6 Gel filtration chromatography of proteins in the sword bean extract and $MgCl_2$ mixture.

$MgCl_2$ was added to the sword bean extract at a final concentration of 60 mM. The $MgCl_2$ mixture was separated into the supernatant and precipitate by centrifugation. Supernatant proteins (2 mL) were loaded onto a gel filtration column that was equilibrated with 60 mM $MgCl_2$ at a flow rate of 0.5 mL/min. Eluted samples were collected in volumes of 1.5 mL. (A) The eluted samples were reacted with Bradford dye reagent, and then measured at 595 nm. Black bars indicate fractions analyzed by SDS-PAGE. (B) The loaded (L) and eluted samples were subjected to SDS-PAGE (10% polyacrylamide). Numbers represent fraction numbers in panel A. Eluted samples were distinguished between fraction numbers 23–39 and 40–47 on different gel plates.

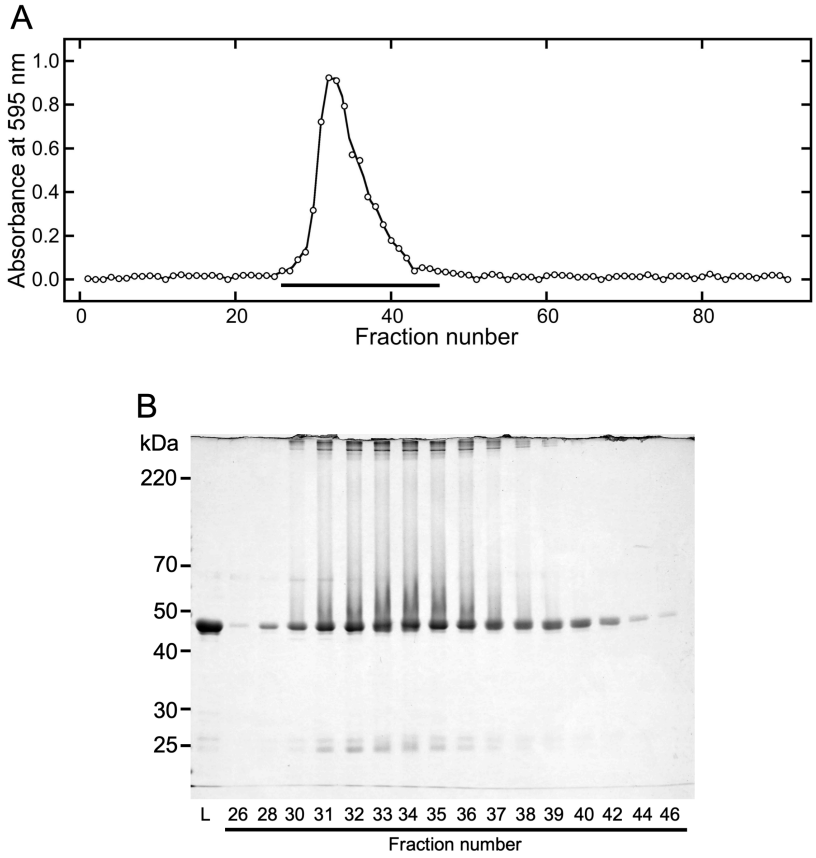

Figure 4.7 Gel filtration chromatography of proteins in the suspension with $MgCl_2$. Canavalin in the sword bean extract was precipitated via the addition of $MgCl_2$ at a final concentration of 15 mM. Precipitated canavalin was suspended in 60 mM $MgCl_2$, and the suspension was separated into the supernatant and precipitate by centrifugation. Supernatant proteins (2 mL) were loaded onto a gel filtration column that was equilibrated with 60 mM $MgCl_2$ at a flow rate of 0.5 mL/min. Eluted samples were collected in volumes of 1.5 mL. (A) The eluted samples were reacted with Bradford dye reagent and then measured at 595 nm. Black bars indicate fractions analyzed by SDS-PAGE. (B) The loaded (L) and eluted samples were subjected to SDS-PAGE (10% polyacrylamide). Numbers represent fraction numbers in panel A.

4.4. Discussion

In this study, I found that the quaternary structure of soluble canavalin in sword bean extract differed with high concentrations of salts. This is the first study, to my knowledge, to describe canavalin as a soluble and unstable monomer in sword bean crude extract. Furthermore, this is the first report to show that the quaternary structure of canavalin changes from a monomer to a trimer in the presence of high concentrations of NaCl (Figure 4.4) and $MgCl_2$ (Figure 4.6). The ionic strength of 200 mM NaCl (0.20) is similar to that of 60 mM $MgCl_2$ (0.18), implying that the trimer form is induced by changing the ionic strength. This result indicates that increasing ionic strength is an important factor for trimer formation. In addition, it is known that globulins are insoluble in deionized water but dissolve in dilute salt solutions. This evidence indicates the possibility that the specific property of globulins is derived from the ion strength-dependent alterations in quaternary structure.

Interestingly, the soluble trimer form of canavalin slowly became aggregated in the presence of 60 mM $MgCl_2$ (Figures 4.6 and 4.7). This aggregation was also observed when 200 mM $CaCl_2$ was added in a preliminary experiment (data not shown). However, in the presence of 200 mM NaCl, aggregation was not observed (Figures 4.4 and 4.5). Previous studies have reported that $MgCl_2$ can precipitate canavalin in the extract; however, NaCl does not have the same effect as shown in Chapter 2. In a similar process involving the formation of tofu-like precipitates salt bridges formed via divalent cations trigger the aggregation of soybean proteins (Arii and Nishizawa, 2018), which are also classified in the globulin class, along with canavalin. Hence, the different properties observed with canavalin following the addition of different salts might also be induced by the formation of salt bridges through divalent cations triggering the aggregation of the

trimer form of canavalin in the presence of $MgCl_2$.

A previous result as shown in Chapter 2 reported that $MgCl_2$-precipitated canavalin can become solubilized in the presence of high concentrations of salts but not in distilled water. These phenomena raise the question of why the monomeric form is soluble in extract. The extract would contain specific minerals, as estimated by Mohan and Janardhanan (1994), to be approximately 0.32 mM sodium, 63 mM potassium, 73 mM calcium, and 3.4 mM magnesium. However, since the extract was prepared after dried beans had been soaked in 10 volumes of distilled water for 18 hours, the true concentrations of these minerals would be much lower than that observed in the previous study. In addition, if these minerals contributed to the solubilization of canavalin in the extract, canavalin would be in the trimer form within the extract. Minerals present with the beans would not contribute to the solubilization of canavalin in the extract. In addition, the trimer form of canavalin might become dissociated in distilled water to the unstable monomer form. However, since the $MgCl_2$-precipitated canavalin would be more stable than the soluble monomer form, the $MgCl_2$-precipitated canavalin would not be solubilized in distilled water. To understand the relationship between structure and solubility, it would be important to compare the secondary structure of the monomer with that of the trimer. However, it is difficult to isolate the monomer because it immediately changes to a trimer or precipitated form under various conditions. For this comparison, I must find the condition that can keep canavalin in its monomeric form.

Canavalin was the first protein to be isolated from jack beans (Sumner and Howel, 1919), and its primary structure was later characterized in both the sword bean (Yamauchi *et al*., 1988; Takei *et al.*, 1989) and jack bean (Ng *et al.*, 1992; Ng *et al.*, 1993) using cDNA sequencing. In previous studies, jack bean canavalin was purified and

crystalized in the trimer form in the presence of high concentrations of NaCl (Ko *et al.*, 1993b; Ng *et al.*, 1993; Ko *et al.*, 2000). Although three-dimensional structures of canavalin from sword beans have not yet been determined, the primary canavalin structures from both species differ by only 2 of the 419 amino acids; they would, therefore, be expected to have a high homology in their three-dimensional structures as well. These reports were consistent with the findings of the present study that canavalin exists in a trimer form in the presence of higher concentrations of NaCl (Figure 4.4) or $MgCl_2$ (Figure 4.6).

Canavalin is classified as a vicilin from the 7S globulin family. The three-dimensional structure of β-conglycinin, which is classified in the same group, also has a trimer structure (Maruyama *et al.*, 2001). β-conglycinin was also purified and crystallized in the presence of high concentrations of NaCl (Maruyama *et al.*, 2001; Morita *et al.*, 1996). These findings suggest that 7S globulins occur in trimeric form in the presence of high concentrations of NaCl. However, it remains unclear whether 7S globulins are present as trimers in bean seeds. As shown in Figure 4.3, sword bean canavalin in the extract was found to be in the monomer not trimer form. This suggests that canavalin in sword bean seeds is present in the monomer form. Therefore, β-conglycinin in seeds might also be present in monomer form. As reported in Chapter 2, $MgCl_2$-precipitated canavalin was not solubilized in distilled water. In the crude extract, canavalin occurs as a soluble monomer; however, the conditions that enable the occurrence of this form were not elucidated in the present study, and thus require further investigation.

In conclusion, I found that sword bean-extracted canavalin in distilled water occurs as a monomer structure; however, the addition of high concentrations of NaCl or $MgCl_2$ induces a change from the monomeric to the trimeric form. In future studies, I aim

to further investigate the conditions that enable the existence of soluble monomers. These data serve as an important reference to analyze sword bean protein characteristics and 7S globulin characteristics.

Chapter 5

A crude sword bean extract is gelated by cooling

5.1. Introduction

The sword bean (*Canavalia gladiata*) is a leguminous plant that originated in the Asian continent and subsequently spread throughout the tropics. Eaten as a green vegetable in Asia (Purseglove, 1968), this plant has particular agronomic traits, including a high cultivation temperature (15–30°C). Moreover, its average yield is comparable to that of the soybean (Bressani *et al.*, 1987), and it is relatively resistant to pests and diseases (Smartt, 1976). Concerning nutritional properties, sword beans contain approximately 26% protein, 3% fat, and 62% carbohydrate (Vadivel and Janardhanan, 2005). These characteristics make the sword bean of potential use in the preparation of processed foods.

In Chapter 1, I prepared three sword bean extracts (Extract A, B, and C) using my own methods established for this purpose (Figure 1.1). For the preparation of Extract A, soaked beans were ground in distilled water and separated into the extract and waste following heating. Extract B was prepared in the same manner but without heating. Finally, Extract C was produced by heating Extract B and removing the resulting precipitates. In Chapter 1 and 2, I found that Extract B contains a large amount of proteins and its preparation represents an inexpensive and simple method for the purification of the major protein present, canavalin, which has a high leucine content (Yamauchi *et al.*, 1988; Takei *et al.*, 1989). The characteristics of Extract B make it a suitable source for the extraction of proteins, and the other extracts obtained may also be useful for the production of processed foods.

In the present study, I observed that Extract A gelates when cooled. Moreover, I

established a novel method for the extraction of gelling substances from dried sword beans by carefully examining experimental conditions. In addition, the temperature at which these substances gelate and that at which the resultant gel melts were also determined. This work provides information that will contribute to the utilization of the sword bean in the food industry.

5.2. Materials and Methods

5.2.1. Materials

White sword beans were purchased from Morika Beiten (Nara, Japan), and general chemical reagents and starch and cellulose were purchased from Wako Pure Chemical Industries (Osaka, Japan).

5.2.2. Preparation of sword bean extracts

Sword bean extracts (Extract A and C) were prepared according to previously described methods in Chapter 1 (Figure 1.1). Dried sword beans were soaked in 10 volumes (v/w) of distilled water at 20°C for 18 h. The soaked beans were then ground on ice for 5 min in 8 volumes (v/w) of distilled water using a hand blender (CSB-77JBSTRW, Cuisinart, Stamford, CT, USA). The suspension containing ground beans was incubated at 100 or 105°C without stirring in a block bath (CDB-105, AS ONE, Osaka, Japan) for 3 min, or was boiled with gentle stirring on a heated stir plate (RET control-visc, IKA, Staufen, Germany) for 3 min. The ground beans were removed from the heated suspension by sieving through a cotton cloth. The filtrate, which corresponds to Extract A described in previous reports, was separated into supernatant and precipitate by centrifugation at 9,100 × *g* at 20°C for 10 min. In another protocol, the ground beans were first removed from

the suspension by sieving through a cotton cloth prior to the application of heat. The filtrate was boiled as described above, and the mixture was again sieved through a cotton cloth. This filtrate corresponds to Extract C in previous reports and was subsequently separated into supernatant and precipitate by centrifugation at 9,100 × *g* at 20°C for 10 min.

5.2.3. Analysis of gelation

Extracts were prepared as above for the determination of gelation conditions. Samples were incubated at 20 or 4°C for 1 day and separated into supernatant and precipitate by centrifugation at 9,100 × g at 20°C for 10 min. Wet precipitate weights were measured with an electronic balance (HR-120, A&D Company, Tokyo, Japan), and gelation efficiency was calculated as the percentage of the initial sample weight represented by the wet precipitate weight. Data are presented as the average of three independent experiments.

5.2.4. Analysis of dry weight of gelling substance

Samples were incubated at 20 or 4°C for 1 day and separated into supernatant and precipitate by centrifugation at 9,100 × *g* at 20°C for 10 min. The supernatant was removed. The wet precipitate was dried at 105°C for 3 h until a constant weight was reached. The dry precipitate weight was measured with the same balance and was converted into that per 1 g of dried bean. Data are presented as the average of five independent experiments.

5.2.5. Analysis of optimal incubation time for gelation

The suspension containing ground beans was boiled with stirring and used to determine the optimal incubation time for gelation. Samples were incubated at 20 or 4°C for various lengths of time and evaluated as described above.

5.2.6. Analysis of gelation temperature

The suspension containing ground beans was boiled with stirring and used for analysis of gelation temperature. Samples were incubated at various temperatures for 2 days and separated into supernatant and precipitate by centrifugation at 9,100 × *g* at 20°C for 10 min. Wet precipitate weights were measured and gelation efficiency was estimated as above. Data are presented as the average of three independent experiments.

5.2.7. Analysis of gel melting temperature

The precipitates prepared by incubation at 4°C for 2 days were incubated at various temperatures for 5 min, before being centrifuged at 9,100 × *g* at 20°C for 10 min. Wet precipitate weights and gelation efficiency were then calculated as above. Data are shown as the average of three independent experiments.

5.2.8. SDS-PAGE

Samples were mixed with 0.33 volumes of SDS sample buffer (0.25 M Tris-HCl [pH 7.0], 4% SDS, 5% 2-mercaptoethanol, and 40% glycerol) and incubated at 100°C for 5 min. SDS-PAGE was carried out on 10% polyacrylamide gels at a constant current of 12.5 mA for 2.5 h according to the standard method described by Laemmli (1970). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250. The molecular weight standard was

purchased from Life Technologies (Tokyo, Japan).

5.2.9. Iodo-starch reaction

The iodo-starch reaction was carried out according to the method described by Reid *et al.*, with minor modifications (1982). Various sword bean extracts were prepared using different procedures. Soaked beans were ground in distilled water as described above, before being separated into supernatant and precipitate by centrifugation. The supernatant was then tested directly or following boiling. In a separate protocol, suspensions containing ground beans were boiled and then sieved through a cotton cloth, as described above. The filtrate was subsequently separated into supernatant and precipitate, also as above. A suspension of 1% starch in distilled water served as a positive control. To each supernatant, distilled water as a negative control, and the positive control, 0.025 volumes of iodine-potassium iodide solution was added and the reactions were mixed well.

5.2.10. Statistical analyses

The t-test was used to compare means among groups in the experiment testing the effect of incubation temperature and in the experiment for dry weight. For the extract procedure and extract incubation temperature experiments, a one-way analysis of variance and Bartlett test were employed to compare group means. Post hoc analysis was performed with the Tukey–Kramer test when analysis of variance indicated a significant difference. Differences associated with p values < 0.05 were considered statistically significant.

5.3. Results

5.3.1. Gelation of sword bean extract

Sword bean extract was prepared by boiling with ground beans present, before sieving through a cotton cloth. The extract was then incubated at 20 or 4°C for 1 day (Figure 5.1). The extract initially appeared slightly cloudy (Figure 5.1A, a); however, after incubation at 4°C, it became opaque and solidified (Figure 5.1A, c). Incubation at 20°C did not have this effect (Figure 5.1A, b). Following centrifugation of these incubated samples, gelation efficiency was calculated to be 15.3 ± 0.3% for incubation at 4°C, and 1.8 ± 0.3% for that at 20°C (Figure 5.1B), and this difference was found to be significant ($p < 0.001$). These results show that sword bean extract can be gelated by incubation at 4°C, representing the first report of the gelation of an extract from this plant.

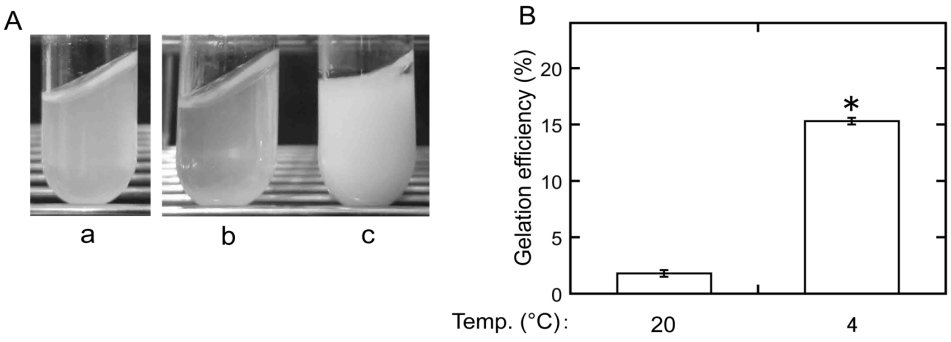

Figure 5.1 Gelation of sword bean extract.

(A) Soaked sword beans were ground in distilled water. The suspension containing ground beans was then boiled and sieved through a cotton cloth (a). The extract was incubated at 20°C (b) or 4°C (c) for 1 day. The photographs of the extracts were taken while inclining the test tubes at 45°. (B) After incubation, the gelation efficiency (the weight of the wet precipitate as a percentage of that of the initial sample) was calculated. Data are expressed as the means ± standard deviations of three independent experiments. The statistical significance of differences was determined by the *t*-test. *$p < 0.001$.

In addition, the incubated samples were dried following the removal of supernatant. The dry weight was calculated to be approximately 71.5 ± 0.4 mg/g dried bean for incubation at 4°C and 8.1 ± 0.2 mg/g dried bean for that at 20°C ($p = 0.730 \times 10^{-17}$). The results indicate that gelling substance is extracted in the dry weight of approximately 63 mg/g dried bean. However, since the gelling substance is crude, the dry weight is a rough indication.

5.3.2. Effects of extraction procedure on gelation

To investigate the effects of heating conditions on gelation, extracts were prepared using various heating methods, before being sieved through a cotton cloth (Figure 5.2). Only when the extract was incubated at 4°C after boiling with stirring did gelation efficiency dramatically increase (to 14.1 ± 0.5%, $p < 0.001$). When the extract was incubated at 20°C, heating conditions had little effect on gelation efficiency, which was 1.1 ± 0.2% for heating at 100°C, 0.8 ± 0.0% for heating at 105°C, and 1.4 ± 0.3% for boiling with stirring. In addition, when the extract was prepared by heating at 100 or 105°C before being incubated at 4°C, gelation efficiency was negligible, being 0.8 ± 0.3% for the former and 1.0 ± 0.5% for the latter. These results indicate that boiling is an essential factor in the preparation of gelling substances from sword beans.

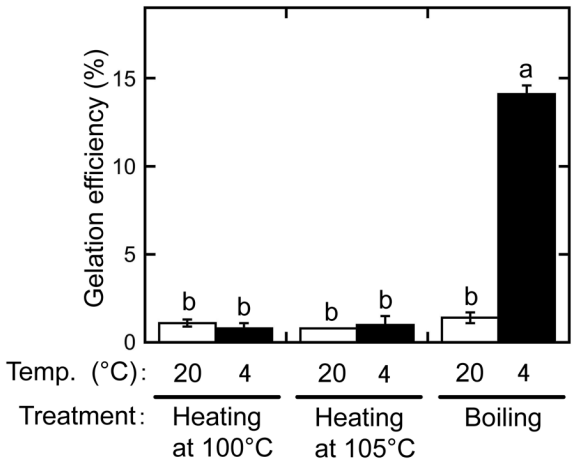

Figure 5.2 Effects of heating treatment during extract preparation on gelation.
Sword bean extracts were prepared by incubating the suspension containing ground beans at 100 or 105°C for 3 min or boiling it with stirring for 3 min. The heated suspension was sieved through a cotton cloth, and the extracts were incubated at 20 or 4°C for 1 day. Gelation efficiency was calculated as in Figure 5.1. Data are expressed as the means ± standard deviations of three independent experiments. The statistical significance of differences was determined by one-way analysis of variance and the Tukey–Kramer test. Different letters indicate a significant difference ($p < 0.001$).

In a previous study, I demonstrated that the extract produced by removing the ground beans before applying heat contains a large amount of proteins, most of which are precipitated by heating at temperatures above 90°C. In the procedures described above, the extract was prepared by boiling without first removing the ground beans. To investigate whether an extract prepared as in a previous study can be gelated after boiling, the ground beans were removed by sieving through a cotton cloth before boiling, after which, the extract was incubated at 4°C and centrifuged (Figure 5.3). Interestingly, this extract did not form a gel when incubated at 4°C, with a gelation efficiency of 1.1 ± 0.0%. In contrast, the gelation efficiency of the extract prepared by boiling with ground beans present was significantly increased by incubation at 4°C (11.6 ± 0.5%, $p < 0.001$). These

results indicate that retaining ground beans during boiling is essential for the preparation of gelling substances from sword beans.

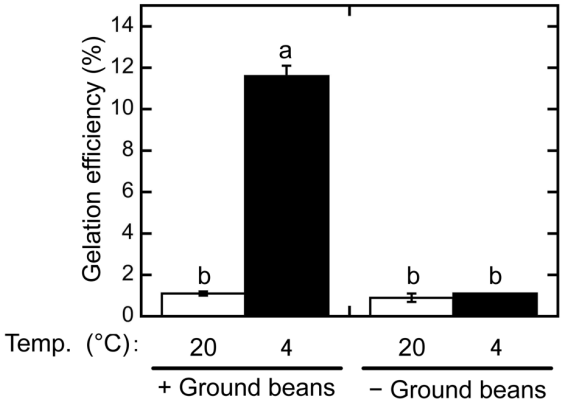

Figure 5.3 Difference between extracts produced by removing ground beans before and after boiling.

One extract was produced by boiling a suspension containing ground beans, which were then removed by sieving (+). Another was prepared by removing the ground beans from the suspension before boiling, after which, it was sieved again (−). The extracts were then incubated at 20°C (white bars) or 4°C (black bars) for 1 day. Gelation efficiency was calculated as in Figure 5.1. Data are expressed as the means ± standard deviations of three independent experiments. Statistically significant differences were determined by one-way analysis of variance and the Tukey–Kramer test. Different letters indicate a significant difference ($p < 0.001$).

In my previous work, I identified that certain seed-derived proteins form a gel when cooled to 4°C following heating (Takenaka *et al.*, 2010). Thus, in the current investigation, I analyzed the proteins in each sword bean extract by SDS-PAGE (Figure 5.4). Prior to boiling, the extracts contained large amounts of protein, whether the ground beans were present (lane 1) or not (lane 2). In contrast, extracts contained few proteins after boiling (lanes 3-6), also regardless of the inclusion of ground beans in the boiling mixture (lanes 3 and 4). As shown in Figure 5.3, gelation of the extract was dependent on inclusion of the ground beans during boiling. These results indicate that proteins are not the main components of the gelling agents in sword beans.

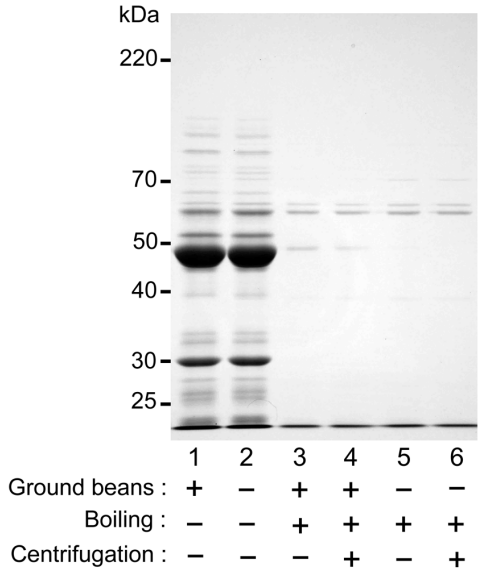

Figure 5.4 Analysis of sword bean proteins in extracts prepared using different procedures.

The effects of each extraction protocol on sword bean proteins were analyzed by SDS-PAGE. During extraction, suspensions containing ground beans (+) (lanes 1, 3, and 4) or not (−) (lanes 2, 5, and 6) were boiled (+) (lanes 3-6) or not (−) (lanes 1 and 2). The boiled suspensions were then sieved to obtain extracts, whereas the unboiled suspensions were not, instead being used directly as extracts. In addition, the extracts were centrifuged (+) (lanes 4 and 6) or not (−) (lanes 1-3, and 5).

5.3.3. Effect of incubation conditions on gelation

To determine the optimal incubation time for gelation, the extract prepared by boiling with ground beans was incubated at 4 or 20°C for 2 weeks (Figure 5.5). The gelation efficiency of the extract incubated at 4°C markedly increased (to 27.2 ± 1.1%) after 1 day, and reached a maximum of 32.5 ± 0.5% after 2 days, before plateauing (Figure 5.5, open circles). In contrast, the gelation efficiency of the extract incubated at 20°C only slightly increased (to 5.8 ± 1.6%) after 14 days (Figure 5.5, closed circles). These observations indicate that maximum gelation of sword bean extract is achieved after 2 days.

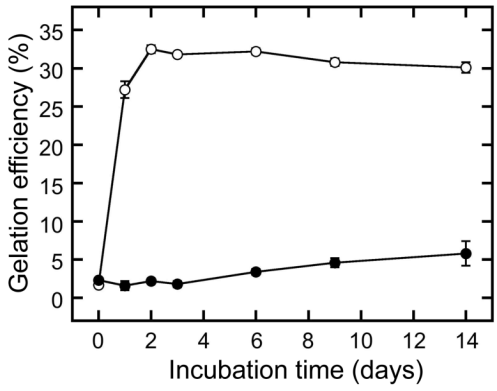

Figure 5.5 Effect of incubation time on gelation.

An extract was prepared by boiling and subsequently sieving a suspension containing ground beans. The extract was then incubated at 4°C (open circles) or 20°C (closed circles) for between 0 and 14 days. Gelation efficiency was calculated as in Figure 5.1. Data are expressed as the means ± standard deviations of three independent experiments.

As described above, the extract was gelated by cooling to 4°C. As gelation temperature is an important factor in food processing, I determined the maximum temperature at which gelation could be induced (Figure 5.6). Each extract was incubated at a temperature between 4 and 20°C for 2 days. Gelation efficiency was considerably higher at 10°C (14.6 ± 0.2%) than at higher temperatures, and was gradually increased by decreasing the temperature (being 21.9 ± 0.1% at 4°C). These changes in gelation efficiency demonstrate that sword bean extract gelates when incubated at less than 10°C, and that this process is promoted by further decreasing the incubation temperature.

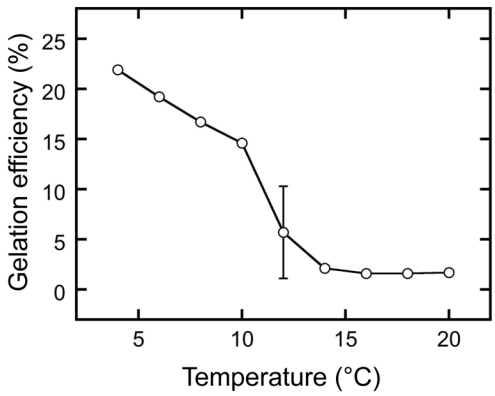

Figure 5.6 Effect of temperature on gelation.

Extracts were prepared by boiling and then sieving suspensions containing ground beans. Each was then incubated at a temperature between 4 and 20°C for 2 days. Gelation efficiency was calculated as in Figure 5.1. Data are expressed as the means ± standard deviations of three independent experiments.

5.3.4. Gel melting temperature

Of the various physicochemical properties of gels, melting temperature is also of importance in the production of processed foods. I therefore investigated the melting temperature of the gel (Figure 5.7) prepared from sword bean extract by incubation at 4°C for 2 days. The gel was incubated at a temperature between 20 and 100°C for 5 min. Gelation efficiency was substantially lower at 65°C (6.0 ± 3.2%) than at 4°C (21.9 ± 0.1%), and plateaued at approximately 2% at 70°C and above. These findings indicate that the gel obtained melted when exposed to temperatures greater than 65°C.

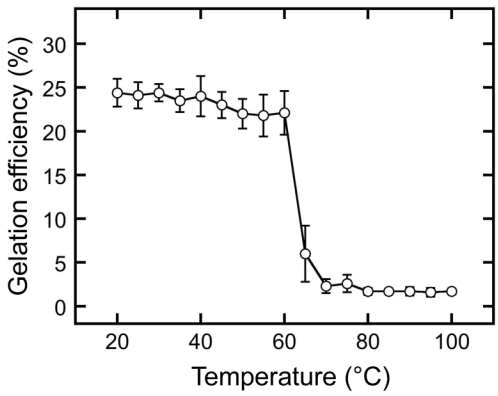

Figure 5.7 Establishment of gel melting temperature.

Gels were prepared from sword bean extracts by incubation at 4°C for 2 days. Each was then incubated at a temperature between 20 and 100°C for 5 min. Gelation efficiency was calculated as in Figure 5.1. Data are expressed as the means ± standard deviations of three independent experiments.

5.3.5. Iodo-starch test of sword bean extracts

Various sword bean extracts were analyzed with the iodo-starch reaction (Figure 5.8). No reaction was observed using the supernatant of the sword bean extract from which the ground beans had been removed (Figure 5.8b), even after it had been boiled (Figure 5.8c); however, slight coloration was noted using the extract prepared by boiling with the ground beans present (Figure 5.8d), i.e., that which gelated when cooled to 4°C. In contrast, strong color development was apparent when an unboiled 1% starch suspension was tested (Figure 5.8e). Given the data depicted in Figure 5.1, B, which show a gelation efficiency of 15.3 ± 0.3%, it seems likely that the substances responsible for gelation represent more than 1% of the extract. Thus, these substances are unlikely to be starches.

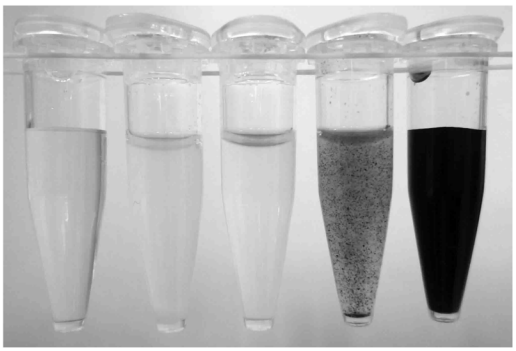

Figure 5.8 Iodo-starch reactions to sword bean extracts prepared using different procedures.

Soaked sword beans were ground and separated into supernatant (b) and precipitate by centrifugation. The supernatant was then boiled (c). Separately, an extract was prepared by boiling suspensions containing ground sword beans and sieving them through a cotton cloth (d). Each extract, distilled water (a), and 1% starch (e) were then mixed with iodine-potassium iodide solution.

5.4. Discussion

Gel-forming molecules are utilized in various processed foods as gelation agents, stabilizers, and thickeners. For instance, gelatin, agar, pectin, and carrageenan are used as thickening stabilizers in jelly, pudding, dressing, sauce, and yogurt, among other products. In this study, I found that sword bean extract gelated at 4°C, representing the first report that this bean is a source of plant-derived gelling agents.

Gelling substances are broadly separated into proteins and polysaccharides. Examples of the former include proteins from egg white (Funaki *et al.*, 2017), soybean (Arii and Takenaka, 2013; Arii and Takenaka, 2014), sesame, and perilla (Takenaka *et al.*, 2010; Takenaka *et al.*, 2011), and those of the latter include agar (Tako, 2015), pectin (Abid *et al.*, 2017), and carrageenan (Tako, 2015). This begs the question of whether the major gelling substance in sword bean extract is a protein or a polysaccharide. SDS-PAGE results in this study indicated that few proteins were present in the boiled sword bean extract (Figure 5.4, lanes 3 and 4). In addition, a previous study in Chapter 1 demonstrated that most sword bean proteins are removed from the extract by centrifugation following heating at more than 90°C. However, gelation efficiency was approximately 15% when the extract was boiled with stirring in the present work (Figure 5.1). It is unlikely that a small amount of protein would result in such high gelation efficiency, suggesting that the major gelling agent is non-proteinous. The preliminary experiments suggest that the gelation substances are polysaccharides (unpublished data); however, extracts of higher purity are required to determine their chemical composition.

Sword beans contain abundant carbohydrates (Vadivel and Janardhanan, 2005; Adebowale *et al.*, 2006), and the starch yield from the dehulled seed is 31% (Adebowale *et al.*, 2006). To explore the possibility that the major gelling substance is starch, sword

bean extracts were analyzed using the iodo-starch reaction (Figure 5.8). Only the extract that gelated after cooling to 4°C (with a gelation efficiency of approximately 15%) exhibited slight coloration (Figure 5.8d). However, the strength of this reaction was far lower than that observed with 1% starch (Figure 5.8e). It does not seem feasible that a gelation efficiency of 15% could result from the presence of starch at a concentration of less than 1%. Moreover, an iodo-starch test of boiled starch solution (Figure 5.9c) resulted in a color similar to that observed with untreated starch (Figure 5.9b and c). These results imply that the major gelling substance is not starch and is not detectable using the iodo-starch reaction. In addition, these results show that little sword bean starch was extracted using my methods. I also examined the possibility that cellulose, a typical plant-derived polysaccharide, is the main sword bean component responsible for gelation (Figure 5.9d and e). Neither the untreated nor the heated cellulose sample reacted visibly in the iodo-starch test. Furthermore, cellulose is almost insoluble in water, even when boiled (Olson *et al.*, 1987). As shown in Supplemental Figure 5.3, most of the cellulose was in a solid state in these samples, whether boiling was carried out (d) or not (e). In contrast, the sword bean gelling substances dissolved in distilled water when boiled (Figure 5.1). This discrepancy in solubility precludes the possibility that the principal gelling agent is cellulose. Considering my results together, I deduce that the gelling substances are not starches or celluloses, but other polysaccharides.

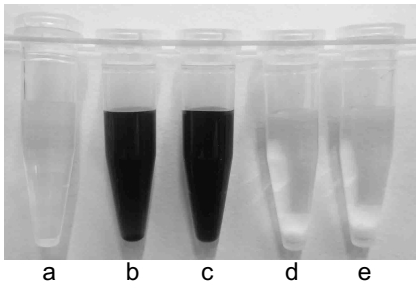

Figure 5.9 Iodo-starch reaction to starch and cellulose.

Distilled water (a) was used as a control. A suspension of 1% starch (b) was incubated at 100°C for 3 min (c). The unboiled 1% starch (b) gave a similar result to that obtained with the same suspension in the test depicted in Figure 5.8, e. A suspension of 1% cellulose was also tested with (e) or without (d) heating.

The gelling substance gelated at temperatures below 10°C (Figure 5.6) and the resulting gel melted at those above 65°C (Figure 5.7). This difference between gelation and melting temperatures demonstrates that the major gelling substance displays thermal hysteresis. In approximate terms, agar gelates at less than 35°C and melts at more than 80°C (Shintani *et al.*, 1975). Thus, the gelation temperature of the gelling substance from sword beans is lower than that of agar, and the difference between its gelation and melting temperatures is larger. In addition, it does not gelate at room temperature (Figure 5.6). Therefore, this gelling agent is suitable for use in food processing. However, the nature of this substance remains unclear owing to the crudity of the extract tested.

Extracts of higher purity are required to identify the substance and determine its characteristics. The first step of the extraction comprised grinding sword beans in distilled water, followed by separation of the extract from the ground beans. My results suggest that the gelling substance derives from the ground beans themselves (Figure 5.3). Separation will be a crucial step for the establishment of an improved procedure for the

extraction of the gelling substance, and is a feasible process from the perspective of food industry applications. I previously reported in Chapter 1 the establishment of a method for the extraction of sword bean proteins, which are found in abundance in the extract that remains after sieving out the ground beans with a cotton cloth. The residual ground beans might thus be considered waste; however, my present report raises the possibility that they may also be of use, particularly for the extraction of gelling substances.

Stirring during boiling appears to be an important factor for the extraction of gelling substances from sword beans, as the different heating conditions tested here brought about different results (Figure 5.2). Cooling resulted in gelation when the sword bean suspension was boiled with stirring, but not when it was incubated at 100 or 105°C without stirring.

In conclusion, I found that a crude sword bean extract could be gelated, representing the first report of a gelling substance being present in this plant. A method of crude extraction was established and the conditions under which gelation of the crude extract occurs were determined in detail. Gelation of the extracted gelling substance occurred at 10°C and the resulting gel melted at temperatures above 65°C. In addition, the presence of ground beans is an essential factor for extraction of the gelling substance by boiling. Although the nature of this substance remains unclear, it is evident that it can be extracted from sword beans in large quantities. In future work, I will prepare high-purity extracts in order to identify this substance, which I expect will be of use in food processing, pharmaceuticals, drug delivery, and tissue engineering, among other applications.

General discussion

Sword bean could be potentially used for processed foods due to its agricultural and nutritional features, though, it has rarely the case. The reason for that is the lack of studies concerning the processing characteristics and nutritional components of sword bean. In this study, I extracted nutritional components to use them as sword bean-derived food materials and revealed their physicochemical properties.

In Chapter 1, a method for sword bean protein extraction was established. Dried sword beans were soaked in 10 volumes of distilled water at 20 °C for 18 h and soaked beans were ground in distilled water. The suspension was squeezed using a cotton cloth and an extract has been separated. This extract contained proteins abundantly (Figure 1.3). When the suspension was squeezed after heating, the proteins were lost due to heat denaturation and were not extracted. I observed that the order of heating and squeezing was important for protein extraction. The extracted proteins were precipitated by heating at temperatures above 90 °C (Figure 1.4). In addition, canavalin was precipitated by the addition of $MgCl_2$ to the sword bean extract (Figure 1.5). It was found that sword bean proteins and canavalin could be easily and safely extracted.

In Chapter 2, $MgCl_2$, $CaCl_2$, and NaCl were added to the sword bean extract at various concentrations to investigate the relationship between salt concentration and canavalin solubility. Canavalin solubility reversibly changed, controlled by divalent Mg^{2+} and Ca^{2+} cation concentrations (Figures 2.3 and 2.4). Nevertheless, canavalin solubility slightly changed in the presence of NaCl at any concentration (Figure 2.2). Therefore, canavalin could be easily and inexpensively purified through divalent cation solubility changes. The primary structure of canavalin has a high leucine content, which might be

useful for the prevention of sarcopenia.

In Chapter 3, I examined how pretreatment differences could affect white sword bean (WSB) and red sword bean (RSB) protein extraction and protein properties. Untreated RSBs absorbed little distilled water during a long soaking period (Figure 3.1). Proteins were extracted with high efficiency from untreated and drilled beans (Table 3.3). The protein concentration and protein quantity data indicated that using untreated WSBs was the most optimal for protein extraction (Table 3.3). Canavalin solubility was controlled by the $MgCl_2$ concentration (Figures 3.4-3.6). However, the behavior was distinctly different between extracts prepared with different pretreatments. The results showed that protein extraction efficiency differed depending on the bean type and pretreatment methods. I found that the bean variety and pretreatment methods were equally important factors for sword bean protein extraction.

In Chapter 4, I described that NaCl supplementation caused the nearly complete solubilization of the $MgCl_2$-precipitated canavalin at high concentrations (Figure 4.2). In addition, I examined the sword bean extract-derived soluble canavalin quaternary structures in the presence of high-concentration salts. Canavalin was present in the monomer form in the sword bean extract in distilled water (Figure 4.3) and the trimer form at high NaCl or $MgCl_2$ concentrations (Figures 4.4-4.7). The structural and functional information on proteins is indispensable for the development of protein-based foods, these data could thus be helpful for the development of sword bean protein-containing foods.

In Chapter 5, the gelling substance was extracted from white sword bean. The soaked beans were ground in distilled water, then a suspension containing ground beans was boiled simultaneously stirred, and separated into extracts. The presence of ground

beans is an essential factor for the extraction of gelling substances by boiling (Figures 5.2 and 5.3). Few proteins were present in this extract (Figure 5.4). In addition, it gelated at temperatures below 10 °C, and the resulting gel melted at temperatures above 65 °C (Figures 5.6 and 5.7). It was suggested that the sword bean-derived gelling substances could be used for food processing, pharmaceuticals, drug delivery, and tissue engineering, among other applications.

Sword bean has processing characteristics; sword bean proteins are precipitated by heating or adding divalent cations (Figures 1.4 and 1.5), and gelling substances are gelated by cooling (Figure 5.1). Other beans also have processing characteristics, and many foods are produced using the processing characteristics. For example, tofu is made from soybean through the property that proteins are precipitated by divalent cations (Arii and Takenaka, 2013). Glass noodles are made from mung bean through the gelatinization of starch (Hosokawa and Saigusa, 1969). Therefore, the findings on the processing characteristics of sword bean in this study could contribute to its further us in processed foods, food materials, and food additives.

Sword bean also exhibits high nutritional value. Canavalin contains abundant leucine that is one of the branched-chain amino acids as described in Chapter 2. A previous study showed that leucine consumption, in combination with exercise, is effective in the maintenance and promotion of muscle mass and strength (Katsanos *et al*., 2006), canavalin could thus potentially help preventing sarcopenia. Moreover, the sword bean-derived gelling substances extracted were not rich in protein (Figure 5.4) but they were likely to be polysaccharides (Figure 5.8). Generally, grains have a high dietary fiber content and soluble dietary fiber has gelling properties (Tsuji, 1990), it could thus be inferred that the sword bean-derived gelling substances contain soluble dietary fiber.

Soluble dietary fiber leads to alterations in the intestinal microbiota, which contributes to hypocholesterolemic effects (Sun *et al*., 2019). Sword bean has such nutritional characteristics, we would thus be able to enjoy these advantages through the use of sword beans. In addition, new health- and care foods that have functions and help swallowing could be produced by combining sword bean proteins, canavalin, and gelling substances.

I also investigated canavalin solubility to reveal its physicochemical properties. Canavalin was insoluble by divalent cations at low concentrations (Figures 2.3 and 2.4). In tofu production, it has been reported that when divalent ions are added to soy milk, divalent ions bind to phytic acid in soy milk. Soy proteins are then insoluble because of the decrease in pH due to the presence of hydrogen ions (Ono *et al*., 1993). Beans have a high content of phytic acid, and sword bean has phytic acid of 7.02 mg/g raw whole seed (Sasipriya and Siddhuraju, 2013). It is surmised that canavalin was insolubilized by the decrease in pH due to the binding of divalent cations and phytic acid in the presence of divalent cations at low concentrations. However, canavalin was solubilized by divalent cations at high concentrations (Figures 2.3 and 2.4). It is also surmised that the solubilization of canavalin is caused by increasing ion strength in the presence of divalent cations at high concentrations.

Canavalin is a globulin protein (Sumner *et al*., 1983), similar to soybean proteins, β-conglycinin, and glycinin (Maruyama *et al*., 2001). Globulin proteins are soluble in a salt solution but canavalin, β-conglycinin, and glycinin are extracted in distilled water (Figure 1.3). It is reported that because a lot of salts are contained in legumes (Circle, 1941). Legumes have high protein content among grains, and studies on the structure and function of leguminous proteins have been conducted to use their proteins for foods. These reports on canavalin could contribute to the study of other 7S globulin proteins.

In conclusion, I examined the processing methods of sword bean, a food material with an unexploited use. I showed that sword bean exhibits various processing characteristics and nutritional functions and its use in the food industry could thus be potentially interesting. Furthermore, I indicated the physicochemical properties of the sword bean protein canavalin. The scientific results presented here could be provide important additional information to study other leguminous proteins and 7S globulin proteins. In addition, scientific information on the structure and function of proteins is indispensable for the development of plant protein foods. Increasing the number of choices for food materials makes our diet more abundant. I hope that this study will contribute to enriching our dietary lifestyle and creating new food cultures.

Summary

Protein supply source currently depends on domestic animal meat to a large extent, we thus took multiple animal-based foods. However, recent studies showed that the consumption of animal-based foods increases the risk of cancer. Nevertheless, the consumption of plant-based foods could inhibit the development of foci and decreases the risk of cancer. These reports imply that plant-based food intake is important for our healthy lifestyle. We depend on soybean for the most source of plant-based foods today. However, in recent years, soybeans have been used as materials for oils and fats. The oils and fats from soybeans are used as biodiesel fuel, which is an alternative to gas oil for environmental conservation. Therefore, soybean is in great demand. However, its yield varies quite due to climate changes caused by global warming or abnormal weather. To deal with these problems, I considered the use of other beans for the supply of plant-based foods instead of soybean. From the nutritional and agricultural characteristics shown below, I selected sword bean (*Canavalia gladiata*) as the first candidate for the development of new plant-based foods.

Sword bean (*Canavalia gladiata*) is a leguminous plant originating in the Asian tropics and subtropics. This plant grows even at high temperatures (15–30 °C), and its average yield is comparable to that of soybean. Moreover, it is relatively resistant to pests and diseases. Sword bean has been eaten as a green vegetable in Asia and used as a source of Chinese medicines in many East Asian countries. In Japan, sword bean-containing pods have been used for industrial supplies, teas, or different kinds of toothpastes. Regarding nutritional properties, sword bean contains approximately 61.8 % carbohydrate, 25.5 % protein, and 3.3 % fat.

As described above, a new source of plant-based foods would be much required. Based on the agricultural and nutritional features of sword bean, it exhibits the potential to be used for foods. However, it has rarely been used for processed foods. The reason for that is the lack of studies about processing characteristics and nutritional components on sword bean. In this study, I attempted to extract nutritional components to use them as sword bean-derived food materials and examined their physicochemical properties.

In Chapter 1, a method to extract sword bean proteins was established. Soaking time was determined by measuring the size and weight of the soaked beans. From the observed changes in sword bean size and weight, sword beans were soaked for 18 h. The soaked beans were ground in distilled water. The suspension containing ground beans was heated and then separated into an extract (Extract A) and waste by squeezing with a cotton cloth. In another manipulation, the suspension was squeezed to be separated into an extract (extract B) and waste. Furthermore, extract B was heated and then separated into an extract (Extract C) and waste by squeezing. Extract B contained more protein than Extract A and C. It was found that the sword bean proteins precipitated due to heat denaturation. The extracted proteins were precipitated by heating at temperatures above 90 °C. In addition, canavalin was precipitated by adding $MgCl_2$ to the sword bean extract. It was found that sword bean proteins and canavalin could be easily and safely extracted.

In Chapter 2, $MgCl_2$, $CaCl_2$, and NaCl were added to Extract B at various concentrations to investigate the relationship between the concentration of salts and canavalin solubility. When $MgCl_2$ was added to extract, the supernatant canavalin was most reduced at 16 mM and was increased, reaching a plateau at 50 mM. When $CaCl_2$ was added to the extract, the supernatant canavalin completely disappeared at 10 mM and was increased, reaching a plateau at 140 mM. However, canavalin solubility was slightly

changed in the presence of NaCl at any concentration. In addition, canavalin solubility was reversibly changed controlled by the divalent cation, Mg^{2+}, and Ca^{2+} concentration. The primary structure of canavalin has a high content of leucine, which might be useful for the prevention of sarcopenia. Canavalin could be easily and inexpensively purified through the use of solubility changes by divalent cation concentration.

In Chapter 3, I examined the effect of pretreatment differences on protein extraction and protein properties of white sword bean (WSB) and red sword bean (RSB). As shown in Chapter 1, untreated WSBs fully absorbed distilled water by soaking. However, untreated RSBs hardly absorbed water. In addition, I examined the effect of different pretreatments on protein extraction efficiency and sensitivity of canavalin to $MgCl_2$. The suspension prepared through the different pretreatments were as follows: suspension that untreated WSB was soaked and then ground (WU), and each sword beans were soaked and then ground (WD, RD), milled each sword beans were suspended with distilled water (WM, RM) was prepared. The suspension was separated into waste and extract. The extract prepared from WU and WD had a higher level of protein extraction efficiency than the other extracts. SDS-PAGE analysis of each extract showed that protein bands in each extract showed a similar pattern. When $MgCl_2$ was added to each extract at various concentrations, canavalin in each extract was insolubilized at low concentrations and solubilized at high concentrations. However, the change in solubility showed different behavior depending on the pretreatment. From these results, it was clarified that differences in sword bean variants and pretreatments affect the properties of the extracted canavalin.

In Chapter 4, the difference between canavalin extracted with distilled water and canavalin in the presence of high salt concentrations was analyzed from the viewpoint of

the quaternary structure. First, the effect of NaCl concentration on $MgCl_2$-precipitated canavalin by the suspension of $MgCl_2$-precipitated canavalin in various concentrations of NaCl was investigated. Soluble canavalin gradually increased with increasing NaCl concentrations until it reached a maximum in the presence of 180 mM NaCl. In addition, the quaternary structures of soluble canavalin in the sword bean extract and in the presence of high-concentration salts were examined by gel filtration chromatography. Canavalin was present in the monomer form within the sword bean extract with distilled water, whereas canavalin was present in the trimer form in the presence of high concentrations of NaCl or $MgCl_2$. From the reports of X-ray crystallography, it has reported that canavalin is a trimeric protein. This protein crystal is prepared in the presence of salts at high concentrations, which is consistent with my reports.

In Chapter 5, sword bean extract (A, B, and C) were extracted in Chapter 1, and Extract A was gelated by cooling. The conditions of the extraction method for gelling substances were investigated. The soaked beans were ground in distilled water, and then a suspension containing ground beans was heated at 100 °C and 105 °C or boiled and separated into extracts. The extract was stored at 4 °C or 20 °C. The extract prepared by boiling was gelated at 4 °C. In addition, the extract prepared by boiling after removing the ground beans was not gelated at 4 °C. The boiling suspension containing ground beans is an essential factor for the extraction of gelling substances. In addition, it was gelated at temperatures below 10 °C, and the resulting gel melted at temperatures above 65 °C. From the results of SDS-PAGE, few proteins were present in this extract, so the gelling substances were not protein. When extract A was analyzed by the iodine-starch reaction, although the extract A was slightly colored, there was a contradiction between the degree of color formation and the amount of gelling substance; therefore, the gelling substance

might not be starch. It was suggested that sword bean-derived gelling substances could be used for food processing, pharmaceuticals, drug delivery, and tissue engineering, among other applications.

In conclusion, I examined processing methods for sword bean and established a method for the extraction of proteins. It was shown that the sword bean has various processing characteristics and nutritional functions, so the use of sword beans in the food industry could be expected. Furthermore, I indicated the physicochemical properties of sword bean proteins, canavalin. The scientific information could be important knowledge to study other leguminous proteins and 7S globulin proteins. In addition, scientific information on the structure and function of proteins is indispensable for the development of plant protein foods. Increasing the number of choices for food materials makes our diet more abundant. I hope that this study will contribute to enriching our dietary lifestyle and creating new food cultures.

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List of publications

Publications related to this thesis

1. Kaho Nishizawa, Tetsuya Masuda, Yasuyuki Takenaka, Hironori Masui, Fumito Tani, Yasuhiro Arii. (2016). Precipitation of sword bean proteins by heating and addition of magnesium chloride in a crude extract. *Biosci Biotechnol Biochem*. 80, 1623-1631.
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